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Award Number: DAMD17-99-1-9269

TITLE: Therapy of Breast Tumor Cells Overexpressing c-erbB-2/neu

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REPORT DATE: October 2002

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 2002	Annual (1 Oct 00 - 30 Sep 02)

4. TITLE AND SUBTITLE
Therapy of Breast Tumor Cells Overexpressing c-erbB-2/neu

5. FUNDING NUMBERS
DAMD17-99-1-9269

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited.

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

Two major independent barriers against the successful therapy of breast cancer are mutation of the tumor suppressor p53 gene and overexpression of the c-erbB-2/neu gene. However, there is little or no information on how, if at all, these molecular defects together influence therapeutic outcome. Of further concern is the absence of any therapeutic agents that could be used against both defects. The present research project was proposed to address these limitations.

The results from this project indicate that both p53 (wild-type and mutant) and overexpression of c-erbB-2/neu lead to cisplatin resistance, and that the resistance due to wild-type p53 and c-erbB-2/neu overexpression can be circumvented by DACH-acetato-Pt. The fact that under certain cellular context, wild-type p53 can lead to substantially greater resistance to an antitumor agent is a novel finding that may have greater limitations in the treatment of breast cancer. The data further indicate that overexpression of c-erbB-2/neu can interfere with p53 regulation when the DNA damaging agent is cisplatin, but there is no effect on regulation when the damage is induced by DACH-acetato-Pt. This suggests that the novel compound may have clinical utility in the treatment of breast cancer which overexpresses c-erbB2/neu.

14. SUBJECT TERMS
Drug resistance, experimental therapeutics, c-erbB-2/neu Overexpression, p53 function

15. NUMBER OF PAGES
110

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Introduction

Two of the major barriers against the successful therapy of breast cancer are mutation of the tumor suppressor p53 gene and overexpression of the c-erbB-2/neu gene. These conclusions have been drawn from many studies reported, individually for each of the genetic defects, over the last decade. However, there is little or no information on how, if at all, these genes influence one and other in the treatment outcome with therapeutic antitumor agents. The end result is that treatment decisions are made in absence of any objective information to guide the way. Of further concern is the absence of any therapeutic agents that could be used under such circumstances. The present research project was proposed to address these limitations.

During the last two years of support from the US Army, further evidence has been obtained to support the concept that the combination of wild-type p53 and the specific platinum agent DACH-acetato-Pt circumvents resistance due to c-erbB-2/neu overexpression. We have used transfection studies and have gained data from biochemical pharmacologic and molecular biologic investigations to demonstrate that effective treatment of cancers overexpressing c-erbB2/neu is a viable clinical proposition.

Body of Report

During the last two years of the project, Tasks 1, 2, 3, 4, 6, and 7 have been the focus of our investigations. Manuscripts have been written or are in preparation (see Appendix), and, therefore, this report will provide only a brief account and defer to the manuscripts for detailed descriptions and presentations of the data. Abstracts are also included from Proceedings of Meetings.

The results to date have been incorporated into manuscripts #1, 2, and 3, which address Tasks 1, 2, 3, 4, 6 and 7:

Manuscript #1. In this manuscript, we demonstrated that overexpression of the oncogene c-erbB2/neu in the wild-type p53 cell line (MCF-7/HER2-18) induces resistance to cisplatin, but increases sensitivity to DACH-acetato-Pt. In the mutant p53 MDA-MB-435 model, overexpression of c-ebB2/neu did not change cisplatin-induced cytotoxicity, but moderately increased the cytotoxicity to the analog. This suggested that the combination of wild-type p53 and c-erbB2/neu overexpression affords a synergistic increase in the cytotoxicity of DACH-acetato-Pt. The differential response of MCF-7/HER2-18 cells was not due to a change in drug uptake or the adducts formed, which suggested that the oncogene-induced increase in resistance to cisplatin was in fact due to an increase in DNA damage tolerance, and increase in sensitivity to the analog was due to a decrease in the tolerance to the adducts induced by this agent. Examination of p53 and target genes indicated that c-erbB2/neu overexpression inhibited cisplatin-induced p53 phosphorylation (particularly at ser-392) and p21 transactivation, but not mdm2 transactivation. In contrast, DACH-acetato-Pt did not mediate significant phosphorylation at ser-392, which was unaffected by overexpression of the oncogene. Similarly, the substantial transactivations of p21 and mdm2 were not by altered by c-erbB2/neu. In conclusion, it was demonstrated that cisplatin and the analog activate independent signaling pathways, which mediate differential modification of p53 and p53-dependent transactivation of p21 and, thereby, the drug-dependent cytotoxic response. Moreover, the synergistic effect of wild-type p53 and c-erbB2/neu overexpression on the cytotoxicity of the analog was ascribed to a sustained phosphorylation of p53 on serine-15.

Manuscript #2. In manuscript #1, we indicated that differential cytotoxicity of cisplatin and DACH-acetato-Pt may be due to overexpression of c-erbB2/neu and/or differences at the p21 level. We explored this further in manuscript #2, which, in accord with Task 1, describes and characterizes breast cancer cell lines that are in addition to those described in the Year-1 report. An interesting facet of the study to emerge was that not only was DACH-acetato-Pt more effective than cisplatin in the wild-type p53 breast cancer cell lines, but that not all p53-mutant cell lines were resistant to the analog. The greater cytotoxicity of the analog over cisplatin against certain tumor models was associated with their wild-type p53 status and/or an intrinsic overexpression of c-erbB2/neu in combination with basal expression of p21. We selected for further evaluation two such cell lines, both expressing p21 and overexpressing the oncogene, but one with wild-type p53 (ZR75-1) and the other with mutant p53 (T47D). In these cell lines, biochemical pharmacology studies confirmed that the greater cytotoxicity of the analog was due to a reduced ability of cells to tolerate DNA adducts induced by the analog as compared to cisplatin. In both cell lines, dose-depende[Page 5]nt degradation in p21 and cyclin D1 by cisplatin

and the analog was evident. Interestingly, the p21 degradation, but not cyclin D1, could be inhibited by the proteosome inhibitor LLnL. These observations were consistent with cell cycle effects, which interestingly could not be associated with differential drug sensitivity. Nevertheless, it was noteworthy to appreciate the close correlation between p21 basal expression and resistance to cisplatin on the one hand, and sensitivity to DACH-acetato-Pt on the other. Thus, it was concluded that p21, in conjunction with p53 and c-erbB2/neu status, plays a key role in modulating sensitivity/resistance to platinum-based compounds in breast cancer.

Manuscript #3. A major outcome of manuscripts #1 and 2 were the modulatory roles of wild-type p53 and/or overexpression of c-erbB2/neu on the differential cytotoxicity of cisplatin and the analog DACH-acetato-Pt. As a definitive effort to demonstrate the role of these two genes, we attempted to identify and include in our studies a breast tumor model with a characteristic of having null p53 and overexpressing c-erbB-2/neu that could be transfected with a temperature-sensitive (TS) p53 vector. However, we were unsuccessful in this endeavor. Therefore, and as a back-up option, we transfected the ovarian SKOV-3 cell line, which has the required genotype, with the TS-p53 vector. Although the SKOV-3 is of ovarian origin, the etiology of this disease is similar to breast cancer, and therefore the data generated have relevance to breast cancer.

In manuscript #3, we generated stable transfectant clones expressing TS-p53 to test the hypothesis that DACH-acetato-Pt, in conjunction with expression of wild-type p53 in a cell line overexpressing c-erbB2/neu, will circumvent cisplatin resistance. At 37°C, when TS-p53 functions as mutant p53, sensitivity to cisplatin was unaffected when the selected clone expressed a low level of p53 expression, but a high level of p53 expression led to a two-fold increase in resistance. A similar, but less pronounced, response was observed with DACH-acetato-Pt. This suggested that the dominant-negative mutant p53 has a gain-of-function effect on platinum resistance. At 32°C, when p53 functions as wild-type, the sensitivity to cisplatin was comparable between the neo and TS-p53 clones. This was good evidence that with cisplatin, overexpression of c-erbB2/neu was the dominant factor in the resistant phenotype. However, the sensitivity of TS-p53 clones to DACH-acetato-Pt at 32°C was increased independent of the level of p53 expression. Thus, the wild-type p53 function was dominant over the effects of c-erbB2/neu when the agent was DACH-acetato-Pt. These relative effects on cytotoxicity by the two Pt agents were not due to alterations in drug uptake or levels of adduct formed, but were correlated directly to the ability of cells to tolerate DNA damage. Since the relative effect on tolerance may be mediated through phosphorylation of p185 (translation product of c-erbB2/neu) and Akt, both cell survival factors, we exposed cells to the [Page 6]drugs and estimated these proteins by Western analysis. The data demonstrated that cisplatin-induced DNA damage significantly activated the Akt pathway through increased phosphorylation of p185, whereas the moderate increases in phosphorylated forms of p185 and Akt were transient. These results explain in part the observed cytotoxic effects of cisplatin and DACH-acetato-Pt in cell lines overexpressing c-erbB2/neu and mutant or wild-type p53.

Note; Task 5 has been attempted, but because of low sensitivity of the assays, progress has been negligible.

Key Research Accomplishments

- Overexpression of c-erbB-2/neu increases resistance to cisplatin independ[Page 7]ent of p53 status, but increases sensitivity to the analog DACH-acetato-Pt either moderately when p53 is mutant or substantially when p53 is wild-type.

- Resistance to cisplatin is not due to a decrease in drug uptake or a reduction in DNA adducts formed. Conversely, the increase in sensitivity to DACH-acetato-Pt was not due corresponding increases in intracellular drug or adduct levels. In fact, the ability of cells to tolerate higher adduct levels was the main reason for resistance to cisplatin, whereas cells sensitive to the analog demonstrated reduced tolerance to adducts.

- The differential effects of p185, the product of c-erbB2/neu gene, on cytotoxicity of cisplatin and DACH-acetato-Pt appears to correlate with differences in regulation of p53, particularly at serine-392, and the differential effect on the p185-mediated Akt survival pathway. The role of p21 in combination with wild-type p53 function or cerbB2/neu overexpression was of a prognostic value in the cytotoxicity of the analog. This is consistent with a few reports that p21 may play a role in apoptotic events under certain conditions.

Reportable Outcomes

The recent results arising from this project have been presented in abstract forms at the NCI/EORTC/AACR International Meeting in Miama in October/November 2001 and at the Annual Meeting of the American Association for Cancer Research in New Orleans in March 2001. The results have also been written up as manuscripts (See Appendix).

Conclusions

We have generated data that further consolidates evidence that alteration in pathways involving p53 (wild-type or mutant) and overexpression of c-erbB-2/neu lead to cisplatin resistance, and suggests that c-erbB2/neu has a dominant survival effect. This was confirmed by following the phosphorylation status of the p185 and Akt proteins following cisplatin treatment. However, cisplatin resistance is circumvented by DACH-acetato-Pt, and this is associated with p21 expression and a lack of a durable effect on phosphorylated p185 and Akt. Resistance or sensitivity was not due to changes in drug uptake or levels of adducts formed, but were correlated closely with the relative ability to tolerate DNA damage. The data indicate that overexpression of c-erbB-2/neu can interfere with p53 regulation and activates the Akt pathway when the DNA damaging agent is cisplatin, but there is no effect on activated p53 levels and the modest rise in phosphorylated Akt does not persist when the damage is induced by DACH-acetato-Pt. Collectively, these data support the concept that DACH-acetato-Pt is worthy of clinical trials against breast cancer. Effort will be made to complete the remaining Tasks during the the time remaining (6 months) for this project. However, some of the studies (e.g., DNA damage recognition in Task 5 and phosphopeptide mapping in Task 7) have not been successful due to low sensitivity of the assays. Attempts will be made to develop alternative approaches.

Appendix

1. Abstract – AACR?NCI/EORTC International Confere[Page 8]nce (October/November 2001)
2. Abstract – AACR Annual Meeting (March 2001)
3. Manuscrpt #1 – submitted
4. Manuscript #2 – In preparation
5. Manuscript 3# – In preparation

SCIENTIFIC PROCEEDINGS

92nd Annual Meeting of the American Association for Cancer Research

New Orleans, LA
March 24-28, 2001

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The purpose of the AACR Annual Meeting is to present the most timely and significant research results in all of the scientific disciplines relevant to cancer. All scientists studying the causes, diagnosis, treatment, and prevention of cancer will benefit from attending this meeting. After participating in this continuing education activity, the participant should be better able to understand the latest research findings in all areas of cancer research; implement these findings in diagnosis, treatment, and prevention of cancer; and use these findings to make further progress in research efforts.

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This activity has been planned and implemented in accordance with the Essential Areas and Policies of the Accreditation Council for Continuing Medical Education (ACCME) through the joint sponsorship of the Center for Continuing Education, Tulane University Health Sciences Center and the American Association for Cancer Research. The Center for Continuing Education, Tulane University Health Sciences Center is accredited by the ACCME to provide continuing medical education for physicians.

The Center for Continuing Education, Tulane University Health Sciences Center designates this educational activity for a maximum of 48 hours in Category 1 credit towards the AMA Physician's Recognition Award. Each physician should claim only those hours of credit that he/she actually spent in the educational activity.

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A summary of the disclosure information provided by Annual Meeting presenters may be found on pages 971-986 of this *Proceedings*.

Next Annual Meeting: April 6-10, 2002, San Francisco, CA

MAPK signaling pathways: p38, JNK/SAPK, and ERK1/2. We also analyzed the differential expression of pro-apoptotic and anti-apoptotic-related genes in response to cisplatin treatment in our isogenic cell line model. There was a prolonged (up to 12 h) activation of the p38 and JNK/SAPK pathways in response to cisplatin in the sensitive cells (2008). In contrast, the cisplatin-resistant clone (2008.C13) displayed a shorter duration of activation (3 h) of the p38 and JNK/SAPK pathways. The ERK pathway was activated to a similar extent at the early time points in both cell lines. We found that inhibition of the JNK/SAPK and p38 pathways by small drug inhibitors (SB202190) mediate a resistant phenotype to cisplatin and increase survival. Moreover, 3T3 cells with disrupted c-jun (3T3 cJun-/-) are resistant to cisplatin DNA damaging agent compare to the parental 3T3 cells. This indicate that a critical difference in sensitivity versus drug-resistance may be associated with in duration of the activation of the p38 and JNK/SAPK signal transduction pathways and their downstream target genes (e.g. AP-1 target genes), in response to genotoxic agents. Our results are consistent with the hypothesis that prolonged activation of the JNK/SAPK and p38 pathways in response to cisplatin treatment of ovarian cancer cells leads to cell death. Early signal transduction events probably indicate and/or dictate whether the cell will undergo cell division or apoptosis. Cisplatin resistance may be related to attenuation of certain MAPK signals.

#2282 Mitochondrial Permeability Transition in Acquired Resistance to Cisplatin. Arkan Abadi and Gurmit Singh. *Hamilton Regional Cancer Centre, Hamilton, ON, Canada.*

The purpose of this study was to investigate the role of Mitochondrial Permeability Transition (MPT) in the acquired resistance to cisplatin within the 2008 model system (ovarian carcinoma cell-line). The phenomenon of MPT, in which mitochondria become permeable to molecules smaller than 1.5 kD in size, is mediated by the MPT pore and has been implicated in the release of mitochondrial apoptotic factors, such as cytochrome C, into the cytosol during apoptosis. We have optimized a procedure for the determination of the mitochondrial membrane potential ($\Delta\Psi_m$) by measuring the accumulation of the fluorescent dye TMRM using flow cytometry. This was used to observe the elevated $\Delta\Psi_m$ of the cisplatin-resistant variants (C13 cells) in comparison to their cisplatin-sensitive parentals (2008 cells). This elevation in $\Delta\Psi_m$ appears to contribute to the resistant phenotype and the hypothesis that these mitochondrial alterations are due to differential activity of the MPT pore between the resistant and sensitive cell-lines was tested. We observed that MPT pore inhibitors (cyclosporine A, and bongkrekic acid) and activators (atractyloside) were able to alter the $\Delta\Psi_m$ of 2008/C13 cells. In addition, these MPT pore modulators were also able to alter the resistance of 2008/C13 cells to cisplatin. This suggests that MPT activity plays a role in the mitochondrial alterations observed in C13 cells, as well as in the resistant phenotype itself. In conclusion, a novel mechanism by which ovarian carcinomas may acquire resistance to cisplatin has been identified, and modulated using MPT pore ligands. (Supported by Canadian Institutes of Health Research.)

#2283 Interactions of Tris(8-quinolinolato)Gallium(III) (KP46) with Platinum Drugs in Ovarian and Colon Carcinoma Cells. Michael A. Jakupec, Philippe Collery, and Bernhard Klaus Keppler. *Institute of Inorganic Chemistry, Vienna University, Vienna, Austria, and Service de Cáncerologie, Polyclinique Maymard, Bastia, France.*

Gallium(III) is known to have antineoplastic properties, at least partly due to its ability to compete with iron(III) and thus to inhibit iron-dependent processes such as enzymatic reduction of ribonucleotides to deoxyribonucleotides. Moreover, combinations of gallium(III) with hydroxyurea, nucleoside analogues (gemcitabine, fludarabine), taxanes and cisplatin produce synergistic effects which might be exploited in cancer chemotherapy. However, with intravenous administration of gallium(III) salts only suboptimal tissue gallium levels can be achieved due to rapid renal clearance and nephrotoxicity as dose-limiting side effect. Tris(8-quinolinolato)gallium(III) (KP46), a complex with lipophilic ligands, was developed with the aim of improving the bioavailability of gallium(III) and is the first gallium complex to enter clinical trials. This compound not only makes it possible to establish higher tissue gallium concentrations but also exerts a much stronger inhibitory effect on tumor cell proliferation in vitro than gallium chloride at the equimolar dose. In order to provide a basis for the design of clinical trials using combination chemotherapy protocols we investigated the interactions of tris(8-quinolinolato)gallium(III) (KP46) with the platinum drugs cisplatin, carboplatin and oxaliplatin in human cancer cells in vitro by means of the MTT assay using different schedules of drug exposure. Comparison of drug combination effects with single agent activities revealed additive and synergistic interactions both in ovarian (41M, CH1) and in colon carcinoma cells (SW480). We thus conclude that combination schedules of tris(8-quinolinolato)gallium(III) (KP46) with platinum drugs are recommendable for the clinical setting.

#2284 Modulation by HER2/Neu of the Cytotoxicity of Cisplatin and 1r,2r-Diaminocyclohexane-Diacetato-Dichloro-Platinum(IV)(DACH-Acetato-Pt) against Wild-Type p53 MCF-7 Breast Tumor Cells. M. Watanabe, J. Nakamura, K. Mujoo, A.R. Khokhar, and Z.H. Siddik. *The University of Texas M.D. Anderson Cancer Center, Houston, TX.*

Wild-type p53 facilitates drug-induced apoptosis, whereas HER2/neu (HER2) induces resistance to some antitumor agents, including cisplatin, and sensitivity to others. Therefore, the aim was to study the effect of the non-cross-resistant platinum complex DACH-acetato-Pt against MCF-7/HER2-18 (HER2-18) having wild-type p53 and stably-transfected HER2 gene, and a control isogenic MCF-7/neo (neo) cell line. Basal levels of HER2 by Western analysis were 5.4-fold greater in HER2-18 compared to neo cells, and the active phosphorylated-form of HER2 was detectable in HER2-18 cells but not in neo. The HER2-18 model was 2-fold resistant to cisplatin compared to neo (IC_{50}: 0.83 vs. 0.44 μM using continuous drug exposure; 18.2 vs. 9.8 μM using 2-hour exposures). In contrast, the HER2-18 cell line demonstrated significant collateral sensitivity to DACH-acetato-Pt by up to 2-fold compared to neo (IC_{50}: 0.12 vs. 0.24 μM - continuous exposures; 15.0 vs. 22.1 μM - 2-hour exposures). DNA damage tolerance to CDDP was significantly higher in HER2-18 (12 ng Pt/mg DNA) than in neo (5.8), whereas there was no significant difference between the two models exposed to DACH-acetato-Pt (1.9-2.0 ng Pt/mg DNA). Although wild-type p53 and $p21^{Waf1/Cip1}$ (p21) were induced in neo and HER2-18 models after treatment with cisplatin, the induction was significantly less in HER2-18 cells. On the other hand, both protein molecules were similarly induced in the two cell lines after treatment with DACH-acetato-Pt. Interestingly, p53 was phosphorylated at serine-15 in a dose-dependent fashion in neo cells treated with cisplatin, but phosphorylation was suppressed in HER2-18. With DACH-acetato-Pt, this phosphorylation was very low in both cell lines. In conclusion, overexpression of HER2 induces cisplatin resistance by suppressing p53 induction, possibly through down-regulating serine-15 phosphorylation of p53. DACH-acetato-Pt, in contrast, likely activates an independent p53-mediated apoptotic pathway that is facilitated by HER2 by an unknown mechanism. The results indicate that DACH-acetato-Pt may have utility in the management of breast tumors overexpressing HER2 against a wild-type p53 background. (U.S. Army Grant DAMD17-99-1-9269).

#2285 Sensitization of Human Bladder Cancer Cells (647v) to Carboplatin by Anguidine. L. D. Green, M. D. VanPelt, and C. Orr. *University of Arkasas, Pine Bluff, AR.*

Cisplatin is the most widely used anti-tumor agent for the treatment of solid malignancies including bladder cancer. However, its use is limited due to nephrotoxicity and to the development of either intrinsic or acquired drug resistance. Carboplatin a second generation platinum analog has largely circumvented the toxic side effects of cisplatin. However, carboplatin is generally not effective in cisplatin resistant tumors. In order to broaden the anti-tumor spectrum of carboplatin our laboratory has been investigating strategies to sensitize tumor cells to carboplatin. This investigation was initiated to determine if the protein synthesis inhibitor, anquidine, would modulate cellular sensitivity to carboplatin relative to cisplatin on intrinsically resistant 647v (grade II) human bladder transitional cell carcinoma cells. Cells were pretreated with anguidine (1 ug/ml) for 3h followed by exposure to the platinum drugs for 1h or 3h. The end point of drug effect with or without anguidine pretreatment was determined in the SRB assay. Anguidine increased both carboplatin and cisplatin cytotoxicity on 647v cells as shown by a significant reduction in the IC50 values. The enhancement of carboplatin and cisplatin cytotoxicity by anguidine was 6-fold and 4-fold, respectively. However, cisplatin was about 60-fold more potent on 647v cells compared to carboplatin. These results suggest that anquidine may be useful in broadening the anti-tumor spectrum of carboplatin and cisplatin in resistant tumors.

#2286 Evaluation of Oxaliplatin in Combination with Standard Chemotherapy Agents against Pediatric Tumor Cell Lines. S. Weitman, M M. Revilla, R. Moore, P. Juniewicz, and J. Rake. *Institute for Drug Development, C, San Antonio, TX, and Institute for Drug Development, CTRC, San Antonio, TX.*

Oxaliplatin, a platinum compound, was evaluated in combination with vincristine, doxorubicin, cisplatin, topotecan, and dactinomycin against pediatric neuroblastoma (NGP, IMR-32 and SD), rhabdomyosarcoma (RH1, RH30 and RD), and osteosarcoma (HOS, U2-OS and SA-OS) tumor cell lines. These studies were undertaken to determine the drug-drug interaction (e.g., additive, synergistic, or antagonistic) that exists between oxaliplatin and standard agents. Multiple combinations of oxaliplatin with the standard cytotoxic agent were used with a model free design (Laska, et al. Biometrics 50:834, 1994) to describe the type of drug-drug interaction. These studies suggest that an additive pattern of drug-drug interaction was most frequently observed when tumor cells were exposed to oxaliplatin and standard chemotherapy agents. Evidence of a synergistic interaction was observed between oxaliplatin and dactinomycin against the NGP neuroblastoma cell line. An additive/synergistic was noted on several occasions against osteosarcoma cell lines (cisplatin and doxorubicin against HOS; doxorubicin against U2-OS). Only evidence of antagonism was observed when oxaliplatin was combined with vincristine against the RH1 rhabdomyosarcoma tumor cell line. These studies suggest that oxaliplatin which is in multiple clinical trials, could be combined with several cytotoxic agents against a broad-range of pediatric tumor types.

#2287 A SAS Based Program to Evaluate Synergy—Application to Paclitaxel/Cisplatin Combinations. Liang Zhao, Yalan Wang, Jun Zuo, Jeffrey S. Johnson, Andrew L. Johnson, Jessie L.-S. Au, and Guillaume Wientjes. *Ohio State University, Columbus, OH.*

The most widely accepted method to evaluate the interactive effects of drug combinations uses the median effect approach, and is usually performed on specialized software (Adv. Enz. Reg., 22:27, 1984). We developed a new software program to expand the methods of data representation. The program uses the

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FUTURE ANNUAL MEETINGS OF THE AACR

April 6-10, 2002 • San Francisco, CA
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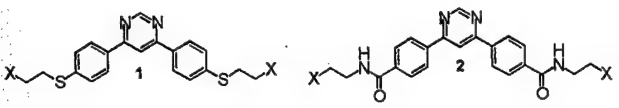
plex melting. Minor structural alteration to the amide 2 inverts this selectivity t also indicates a much weaker association. Furthermore, model building edicts the threading distance of the planar portion of 2 to be a closer match the larger cross-sections of tetraplex DNAs than either duplex or triplex. A vel and versatile synthesis utilising a Suzuki cross-coupling reaction has orded this class of compounds in greatly improved yields and structural versity. Investigations of their interactions with several DNA types will be ported. Structural preferences for DNA binding have been established using uilibrium competition dialysis; binding stoichiometries and thermodynamic rameters have been determined using isothermal titration calorimetry and UV ectrophotometry. These data have been used to inform molecular modelling the complexes between the ligands and duplex, triplex and tetraplex DNA hich may be used as the basis for the design of potential drug compounds hich discriminate elegantly between DNA secondary structures.



#712 Aminoflavone (NSC 686288) induction of cytochrome P450 1A1 in ensitive human tumor cell lines is associated with metabolic activation, NA damage and antiproliferative activity. M. M. Ames, J. C. Schroeder, L. Pobst, M. J. Kuffel, C. R. Arnt. *Mayo Medical and Graduate Schools, Mayo dn, Rochester, MN.*

Aminoflavone (AF) is a potent in vitro and in vivo inhibitor of human tumor cell rowth, and exhibited a unique activity profile in the NCI human tumor cell line creen. The unique pattern suggests a novel mechanism of action. We previ- usly demonstrated extensive cytochrome P450 (CYP)1A1 and CYP1A2 me- abolism of AF by fortified rat and human hepatic microsomes to oxidative etabolites, including an aromatic N-hydroxylamine. We further demonstrated hat sensitive human tumor cell lines exposed to AF converted parent drug to eactive species as detected by covalent binding (MJ Kuffel et al, Proc. Amer. ssoc. Can. Res., 41:370, 2000). We now report that exposure of cell lines from he NCI screening panel to AF results in increased CYP1A1 message (com- ared to untreated cells) as determined by real time RT-PCR in sensitive, but ot resistant lines. CYP1A1 induction correlates with the extent of radiolabeled F covalent binding to macromolecules in those cell lines. DNA damage is uggested by stabilization of p53, increased p21 and interactions of AF with ellular DNA. We also report increased CYP1A1 message and AF covalent inding in three of six low-passage ovarian human tumor cell lines following xposure to AF. These studies support the hypothesis that AF is selectively ytotoxic to human tumor cell lines through induction of CYP1A1, metabolism o cytotoxic species and DNA damage. Additional data will be presented to upport this hypothesis.

#713 Rational design approaches to increase the potency of G-quad- uplex-mediated telomerase inhibitors. M. Read, J. Cuesta-, S. Basra, J. Harrison, A. Reszka, S. Gowan, L. R. Kelland, S. Neidle. *Institute of Cancer Research, London, UK.*

The telomerase enzyme complex is responsible for telomere maintenance in 80-85% of tumours. Inhibition of its action leads to senescence of cancer cells, and eventually apoptosis. We and others have shown that inhibition can be accomplished via folding of the single-stranded telomeric substrate into a higher-order quadruplex structure and thus prevent the hybridisation of the telomere with the endogenous RNA template of the telomerase enzyme. Mol- ecules that can stabilise quadruplexes include anthraquinone, acridine and porphyrin derivatives, with typically telomerase activity in the 1-10 μM range. However most are also effective binders to duplex DNA sequences, and they have cytotoxicities in tumour cell lines that are also in this range. Two lead compounds were synthesised by us with an anilino substituent at the 9-position of the acridine chromophore in addition to substituents at the 3 and 6 positions (Read et al., PNAS, 2001, 98, 4844-4849). These third substituents were pre- dicted by molecular modelling to reside in the third groove of the G-quadruplex. Calculated relative binding energies from molecular dynamic simulations sug- gested an increase in G-quadruplex affinity, whilst duplex-ligand molecular modelling showed that this substituent gave rise to unfavourable steric clashes with duplex DNA. These results correlate well with binding affinities calculated from surface plasmon resonance experiments, and this has been mirrored by a 50-fold increase in the telomerase inhibition potency to levels up to 60nM. We now report a new series of these 3,6,9-trisubstituted acridine inhibitors which have been synthesised to increase interactions in the third groove whilst still maintaining low cytotoxicity. The new analogues were predicted to bind to G-quadruplexes with increased affinity using computer aided drug design (CADD). Incorporating flexible alkyl and cationic sides at the 9-position has produced a significant further improvement in telomerase inhibition potency, to levels up to 12 nM. Their acute cytotoxicities remain low, providing a 1000-fold window between telomerase inhibitory efficacy and cytotoxicity.

#714 Phase I and pharmacokinetic (PK) study on PNU-166196, a novel minor groove binder (MGB) potentiated by glutathione (GSH) and GSH-transferase (GST), administered intravenously (iv) once every 3 weeks to patients (pts) with advanced cancer. M. J. de Jonge, B. ten Tije, A. van der Gaast, F. Fiorentini, C. Fowst, J. Tursi, J. Verweij. *Rotterdam Cancer Institute and Univ Hosp Rotterdam, Rotterdam, Netherlands; Pharmacia Corp, Nerviano, Italy.*

PNU-166196 is a cytotoxic agent that binds to the minor groove of DNA. Of interest, in preclinical studies the cytoxicity of PNU-166196 increased in pres- ence of high levels of GSH and GST. This phase I study was designed to determine the maximum tolerable dose (MTD), dose limiting toxicity (DLT) and PK of PNU-166196, when administered as a 10-min infusion in a 3-weekly cycle. The starting dose, 0.85 mg/m^2 was escalated with an initial accelerated phase (1 pt/dose level) followed by conventional dose escalation in 3-6 pt cohorts after CTC grade 2 toxicity was observed. The PKs of PNU-166196 were assessed after the first and second administration by LC/MS-MS. To date, 28 pts have been treated (median age 55 years, median ECOG PS 1) and 61 cycles are evaluable for toxicity. Dose levels studied were 0.85, 1.7, 3.4, 5.1, 7.5, 10, 12.5 and 15 mg/m^2. DLT consisting of neutropenia (2:2 pts) and thrombocyto- penia with GI bleeding (1:2 pts) were observed at 15 mg/m^2. At expansion of dose level 12.5 mg/m^2 from 3 to 8 patients, uncomplicated prolonged neutro- penia (≥ 7 days) was encountered in 3 patients qualifying for DLT. However, at retreatment at the same dose level the toxicity observed was less severe. At present, dose level 10 mg/m^2 is being expanded to 6 patients. Non-haemato- logical toxicity consists of nausea/vomiting, diarrhea and fatigue and is mainly mild. One partial response was observed in a patient with a chemotherapy refractory GIST. PK data indicate that both C_{max} and AUC increase linearly with dose ranging from 114-3187 ng/mL and 53-2179 ng.h/mL, respectively. The terminal half life was 1-6 hour and the volume of distribution 5-15 L/m^2. Clearance ranged from 6-14 L/h/m^2. According to protocol definitions the recommended dose for further study in this schedule will be PNU-166196 10 mg/m^2 once every 3 weeks. However, current data suggest that under carefully controlled conditions 12.5 mg/m^2 once every 3 weeks may well be feasible.

Summary PK data

Dose mg/m²	0.85 (n=1)	1.7 (n=1)	3.4 (n=3)	5.1 (n=3)	7.5 (n=3)	10 (n=5)	12.5 (n=8)	15 (n=2)
Cmax (ng/ml)	114	195	535 (352-625)	970 (936-1090)	1431 (997-1681)	1997 (1712-2006)	2983 (2023-3187)	2764 (2569-2999)
T½ (h)	0.86	2.12	2.75 (2.15-5.92)	6.06 (4.39-9.05)	4.58 (4.40-5.49)	4.94 (3.43-5.08)	6.13 (4.23-6.46)	4.49 (4.05-4.92)
AUC (ng.h/ml)	53	103	267 (233-345)	466 (408-829)	804 (713-908)	1195 (1194-1311)	1516 (1492-1581)	1979 (1779-2179)
Cl (ml/h/m²)	15963	16352	14554 (9881-14544)	10915 (6153-12534)	9623 (8270-10570)	8324 (7629-8369)	8337 (7905-8465)	7659 (6885-8433)
Vss (ml/m²)	5514	9936	8343 (6992-15541)	9539 (4614-11979)	8300 (6215-8440)	7807 (6261-9075)	6230 (5706-8951)	6410 (6200-6621)

#715 Wild-type p53 circumvents resistance of HER2/neu-overexpress- ing tumor cells in a drug-dependent manner. M. Watanabe, K. Mujoo, P. Hennessey, A. R. Khokhar, Z. H. Siddik. *The Univ of Texas, M D Anderson Cancer Ctr, Houston, TX.*

Wild-type p53 facilitates drug-induced apoptosis, whereas HER2/neu (HER2) induces resistance to some antitumor agents, including cisplatin. We have utilized two models to test the hypothesis that wild-type p53 will overcome drug resistance caused by overexpression of HER2. One model was derived from wild-type p53 bearing MCF-7 breast cancer cells transfected with HER2 (HER2-18). The second model was established by transfecting null-p53 SKOV-3 ovar- ian cancer cells overexpressing HER2 with a temperature-sensitive mutant p53 (TS). To test our hypothesis, we used cisplatin and the novel analog 1R,2R-diaminocyclohexane-diacetato-dichloro-platinum(IV) (DACH-acetato-Pt). HER2-18 cells were 2-fold resistant to cisplatin compared to neo controls (IC50: 0.83 vs. 0.44 μM; continuous drug exposure), whereas they were significantly more sensitive to DACH-acetato-Pt by up to 2-fold (IC50: 0.12 vs. 0.24 μM). DNA damage tolerance to cisplatin was significantly greater in HER2-18 cells (12 ng Pt/mg DNA) than in neo (5.8). Similarly, TS clones were significantly more sensitive to DACH-acetato-Pt compared to neo (IC50: 5.9 vs. 14.3 μM in clone 4; 4.6 vs. 22.3 μM in clone 9) at 32°C, when p53 functioned as wild-type. In contrast, the IC50 of cisplatin at 32°C was unaffected (IC50: 5.8 vs. 5.9 μM in clone 4; 3.6 vs. 4.3 μM in clone 9). DNA damage tolerance to DACH-acetato-Pt was significantly lower in clone TS4 (42.7 ng Pt/mg DNA) than in neo4 (95.1) at 32°C, while there was no significant difference in tolerance to cisplatin. Western immunoblots from the MCF-7 models revealed that in neo cells phosphorylation of p53 at serine 15 and serine 392 contributed to the induction of p53 with cisplatin, whereas a lesser extent of serine 15 and a very poor phosphorylation at serine 392 were seen with DACH-acetato-Pt. Increased levels of HER2 attenuated both the induction of p53 and its phosphorylation at these sites in response to cisplatin, but did not affect them following DACH-acetato-Pt ex- posure. These results suggest that independent pathways are involved in p53 activation for the two platinum agents and HER2 only impinges on the pathway

activated with cisplatin. These results also indicate that an introduction of functional p53 increases sensitivity of cells overexpressing HER2 to DACH-acetato-Pt, but not to cisplatin. Furthermore, the results indicate that introduction of functional p53 in combination with DACH-acetato-Pt may be an effective combination against cells overexpressing HER2. (Supported by the U.S. Army Grant DAMD 17-99-1-9269, and NCI RO1 CA77332 and RO1 CA82361 to ZHS).

#716 The total body clearance of Ecteinascidin 743 is independent of body surface area and other variables related to body size. T. A. Puchalski, G. D. Demetri, R. Garcia-Carbonero, D. P. Ryan, J. W. Clark, D. Harmon, L. Butkiewicz, R. G. Maki, L. Lopez-Lazaro, J. Jimeno, J. G. Supko. *Dana-Farber/Partners Cancer Ctr Harvard Medical Sch, Boston, MA; Memorial Sloan Kettering Cancer Ctr, New York, NY; Clin Research and Development, Pharma Mar, S A, Madrid, Spain.*

Dosages of anticancer drugs are commonly adjusted to the body-surface area (BSA) of each patient to minimize interpatient variability in systemic drug exposure. Ecteinascidin 743 (ET-743) is a cytotoxic marine natural product that has shown promising evidence of activity during early clinical trials against a variety of tumor types including sarcoma and breast cancer. Minimizing variability in the pharmacokinetics of ET-743 between patients appears to be integral to its safe use due to the strong association between AUC and severe toxicity demonstrated in phase I trials. The effect of BSA and other demographic variables related to body size on the total body clearance (CL) of ET-743 were evaluated by retrospectively analyzing a large representative population of adult cancer patients. Pharmacokinetic data was obtained during the first cycle of therapy in a group of 100 patients (54 male, 46 female; median age = 46.6 yr, range = 12.9-77.7 yr) with advanced solid tumors treated in a phase I and several phase II clinical trials. All patients had relatively normal hepatic (bilirubin < 1.5 mg/dl and transaminases ≤ 3-times upper limit of normal) and renal (serum creatinine ≤ 2 mg/dl) function. The drug was administered as a 72-h continuous i.v. infusion to 12 patients at doses ranging from 600 to 1050 $\mu g/m^2$ in the phase I study and as a 24-h continuous i.v. infusion at a dose of 1500 $\mu g/m^2$ to 88 patients in the phase II studies. The concentration of ET-743 in plasma was measured at a single site using an assay based upon high-performance liquid chromatography with electrospray ionization mass spectrometric detection. The sampling schedule used in all trials provided an accurate estimate of AUC by noncompartmental analysis. Values of the uncorrected CL in individual patients ranged from 13.6 to 168.1 l/h with a geometric mean ± S.D. of 65.1 ± 30.8 l/h. BSA values in these patients ranged from 1.2 to 2.8 m^2 with a mean of 1.9 ± 0.3 m^2. Interpatient variability as indicated by the coefficient of variation of the mean was very similar whether the CL was corrected for BSA (47.5%) or uncorrected (47.4%). There was no evidence of a correlation between the uncorrected CL and BSA ($r = 0.13$), total body weight ($r = 0.058$), body height ($r = 0.21$), ideal body weight ($r = 0.20$), and body mass index ($r = 0.13$). Accordingly, there does not appear to be any rationale for normalizing doses of ET-743 to BSA as opposed to administering a fixed dose. Future studies are warranted to prospectively examine the magnitude of intrapatient variability in the AUC following treatment with fixed doses of the drug with the goal of determining the optimal method for dosing this promising new anticancer drug.

#717 Synergistic combinations of brostallicin with other antitumor agents. C. Geroni, T. Colombo, S. Marchini, E. Galliera, M. D'Incalci, M. Broggini. *Pharmacia Corp, Nerviano, Italy; Mario Negri Institute, Milano, Italy.*

Brostallicin (PNU-166196) is a synthetic α-bromoacrylic, second generation DNA minor groove binder (MGB) structurally related to distamycin A, currently in Phase I/II in Europe and US. As previously reported (1,2), brostallicin is active in a number of human and murine cell lines, as well as in murine leukemia and solid tumors xenografts and has shown a therapeutic index in pre-clinical models significantly improved in comparison with other MGBs (7.2 vs 1-2). However, unlike all other cytotoxics, its anti-tumor activity is increased both in vitro and in vivo in the presence of high levels of glutathione (GSH) and glutathione S-transferases (GST) and the GSH/GST system is involved in its mechanism of DNA interaction (3). Multiple combinations of brostallicin with compounds belonging to major classes of antitumor agents were studied. In vivo combination studies have been performed on the basis of brostallicin's newly determined mode of action and ability to overcome several mechanisms of drug-resistance. One hypothesis was to combine brostallicin with agents selecting unresponsive cells due to a high GSH/GST level or with compounds ineffective on mismatch-repair deficient tumors. The model utilized for the primary evaluation of activity/toxicity was the L1210 murine leukemia. Further effective combinations have been tested against human tumor xenografts selected on the basis of the clinical application of the selected drugs. In vivo, on disseminated L1210 leukemia, the combinations with gemcitabine, doxorubicin, CPT-11 and cisplatin have shown additive / more than additive / synergistic antitumor effect. Synergistic antitumor activity has been observed testing brostallicin in combination with cisplatin against a human colon carcinoma (HCT-116) with mismatch-repair deficiency. Further studies are ongoing with other drugs used in clinic. Although the precise mechanism of interaction has not yet been identified, a clear therapeutic gain was observed in preclinical models combining brostallicin with other anticancer agents. These results indicate the value of brostallicin and cytotoxic agents in cancer combination treatment protocols. 1 Geroni C. Proc. Annu. Meet. Assoc. Cancer Res., 91: A1689, 2000. 2 Geroni C. Proc. Annu. Meet. Assoc. Cancer Res., 92: A1759, 2001. 3 Cozzi P. Il Farmaco 56: 57, 2001.

#718 Crystal structure of 9-amino[N-(2-morpholino)ethyl]acridine-4-carboxamide bound to d(CGTACG)2: A DNA complex of a biologically inactive derivative of the acridinecarboxamide topoisomerase poisons. A. Adams, J. M. Guss, W. A. Denny, L. P. Wakelin. *Univ of Sydney, Sydney, Australia; Auckland Cancer Research Ctr, Auckland, New Zealand; Univ of New South Wales, Sydney, Australia.*

The structure of the complex formed between d(CGTACG)2 and 9-amino-[N-(2-morpholino)ethyl]acridine-4-carboxamide, an inactive derivative of the anti-tumor agent DACA, has been solved to a resolution of 1.8 Å using X-ray crystallography. The complex crystallises in the space group P64 and the final structure has an overall R factor of 21.9%. A drug molecule intercalates between each of the CpG dinucleotide steps with its sidechain lying in the major groove, and its protonated morpholino nitrogen partially occupying positions close to the N7 and O6 atoms of guanine G2. The morpholino group is disordered, the major conformer adopting a twisted boat conformation that makes van der Waals contact with the O4 oxygen of thymine T4. A water molecule forms bridging hydrogen bonds between the 4-carboxamide NH and the phosphate group of the same guanine. Sugar rings are found in alternating C3'-exo/C2'-endo conformations except for cytosine C1 which is C3'-endo. Intercalation perturbs helix winding throughout the hexanucleotide compared to B-DNA, steps 1 and 2 being unwound by 10 deg and 8 deg respectively while the central TpA step is overwound by 11 deg. An additional drug molecule lies at the end of each DNA helix linking it to the next duplex to form a continuously stacked structure. The protonated morpholino nitrogen of this "end-stacked" drug hydrogen bonds to the N7 atom of guanine G6, and its conformationally disordered morpholino ring forms a C-H. . .O hydrogen bond with the guanine O6 oxygen. In both drug molecules the 4-carboxamide group is internally hydrogen bonded to the protonated N-10 atom of the acridine ring. Our findings provide new insights into the role of the interaction between topoisomerase II and the drug sidechain in the poisoning of topoisomerase activity by the acridinecarboxamides.

#719 Synthesis, biophysical analysis and molecular modelling of novel diphenylpyrimidine based compounds as potential high-order DNA intercalators. P. M. Murphy, R. T. Wheelhouse, T. C. Jenkins. *Univ of Bradford, Bradford, West Yorkshire, UK; YCR Lab of Drug Design, Univ of Bradford, Bradford, West Yorkshire, UK.*

Tetraplex structures formed in guanine rich DNA by G-tetrads have been shown to be important in the regulation of telomerase activity and cellular replication, whilst triplex DNA stabilisation has been proposed as a binary adduct to "antigene" therapies, targeting and thereby inhibiting gene expression. This comprehensive evaluation of two classes of novel compounds, differing in potential threading distance, clarifies the effects caused by compound structure changes on the interactions and specificities of binding to duplex, triplex and tetraplex DNA. A novel and versatile synthesis utilising a Suzuki coupling reaction has been developed to prepare two classes of novel compounds, based on a previously published diphenylpyrimidine[1], in greatly improved yields. Investigations into their binding interactions with several DNA types have been performed using isothermic titration calorimetry, equilibrium competition dialysis, thermal melting curves, competitive ethidium displacement and DNA-ethidium fluorescence quenching. The compounds have also been evaluated for anti-telomerase activity using the telomerase repeat amplification protocol (TRAP) assay. Binding constants were found ranging from 5.8×10^5 to 1.6×10^6 M(bp)$^{-1}$ for calf thymus DNA and 1.6×10^6 to 4.8×10 M(bp)$^{-1}$ for poly(dT)-poly(dA)•poly(dT) triplex DNA. Equilibrium competition dialysis shows binding selectivity preference to high-order DNA (triplex and tetraplex) over duplex DNA. Thermal DNA melting shows triplex DNA stabilisation (20 °C at [0.5]:[1.0] [drug]:[DNA] ratio) for one class of compounds but no for the other which shows only duplex DNA stabilisation (23 °C at [0.2]:[1. [drug]:[ctDNA] ratio). DNA-ethidium fluorescence quenching indicates a chang in binding mode preference from intercalation of duplex DNA to groove bindi of triplex DNA for one but not the other class of compounds. Telomera inhibition has been shown for both classes of compounds up to an IC_{50} 10μM. Molecular modelling techniques have been applied to obtain ener minimised models of the compounds bound to the high-order DNA targets a these used to explain the DNA structural preferences, binding strengths a stabilisations observed. 1. W. D. Wilson et al, 1988, J. Am. Chem. Soc., 1 8292.

#720 Efficacy of the novel DNA minor groove binding agent, brostallicin, against drug-resistant tumor cells. C. Geroni, S. Marchini, E. Galliera, Broggini. *Pharmacia Corp, Nerviano, Italy; Mario Negri, Milan, Italy.*

Brostallicin, (PNU-166196) is the lead compound of a novel class of antitu agents undergoing Phase I/II clinical trials. The molecule is an α-bromoac amide derivative of four pyrrole units from a distamycin-like frame, in which amidine moiety of distamycin is replaced by a guanidine group. Brostal chemically reacts with a reduced glutathione (GSH) molecule (a reaction c lyzed by glutathione-s-transferase - GST) to give reactive molecules. Th findings suggest that GSH may affect the mechanism of brostallicin-DN teraction and the efficacy, with a potential value in the treatment of tumor with high GSH/GST content. Higher cellular GSH/GST levels have been ported to play a role in the resistance of tumor cells to different drugs, suc alkylating agents, platinum derivatives and anthracyclines. The cytotoxic a ity of brostallicin has been tested on tumor cell sublines resistant to alkyl agents (L1210/L-PAM, L1210/cDDP and L1210/BCNU), to multi-drug r ance (MDR) related drugs (L1210/DX and L1210/TAX) and to camptot

APPENDIX – MS #1 (Submitted)

Differential Modulation by HER2/neu of the Cytotoxicity of Cisplatin and 1R,2R-Diaminocyclohexane-diacetato-dichloro-Platinum (IV) Against Wild-Type and mutant p53 Breast Tumor Cells[1]

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Running Title: Modulation of platinum sensitivity by HER2 and p53

Keywords: HER2/neu, p53, platinum

[1] This work was supported by U.S. Army Grant DAMD 17-99-1-9269, and NCI RO1 CA77332 and RO1 CA82361.

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ABSTRACT

To clarify the effects of HER2/neu overexpression on the sensitivity to cisplatin and the novel analog DACH-acetato-Pt against tumor cells with different p53 status, we have utilized two stable HER2/neu transfection models bearing wild-type or mutant p53. Increased levels of HER2/neu led to an increase in resistance to cisplatin in wild-type p53 MCF-7 cells, but did not affect cisplatin cytotoxicity in mutant p53 MDA-MB-435 cells. On the other hand, HER2/neu overexpression significantly increased sensitivity to DACH-acetato-Pt independent of p53 status, although DACH-acetato-Pt was much more potent against wild-type p53 cells. Biochemical pharmacology demonstrated that the change in cytotoxicity induced by HER2/neu was due entirely to an inverse change in DNA damage tolerance. In MCF-7 cells, both drugs induced p53 in a dose- and time-dependent manner. Overexpression of HER2/neu attenuated both the induction of total p53 and its phosphorylation at serine 15 and 392 in response to cisplatin, but did not reduce p53 induction following exposure to DACH-acetato-Pt. These results suggest that independent pathways are involved in p53 activation for the two platinum agents and HER2/neu only impinges on the pathway activated by cisplatin. DACH-acetato-Pt may have utility in the management of HER2/neu-overexpressing tumors, particularly against a wild-type p53 background.

INTRODUCTION

Two of the major barriers against successful therapy of human cancers are mutation of the p53 tumor suppressor gene and an amplification/overexpression of HER2/neu gene. This is particularly relevant to several tumor types, including ovarian and breast cancers. In invasive breast cancer, for instance, the frequency of mutations in p53 gene ranges from 12-46 %, while HER2/neu is overexpressed in 10 to 34 % (Berns et al. 2000; Ross and Fletcher 1998). Both of these genetic alterations are reported to be powerful predictors not only of survival but also of tumor response to adjuvant therapy (Andersen and Borresen 1995; Burke et al. 1998).

Wild-type p53 is a DNA binding protein, which acts as a transcriptional factor to control the expression of a variety of genes regulating growth arrest and apoptosis (Albrechtsen et al. 1999). In addition to transactivating the cyclin-dependent kinase inhibitor $p21^{waf1/Cip1}$ (El-Deiry 1997), p53 also upregulates MDM2, which binds p53 and acts as an ubiquitin ligase (Honda et al. 1997), and is involved in a negative feedback loop (Momand et al. 2000). Recently, several reports have revealed that p53 protein is activated through extensive post-translational modifications, including phosphorylation and acetylation, in response to stress signals (Lakin and Jackson 1999). Among several phosphorylation sites, serine residue (Ser) 15 prevents the binding of MDM2 resulting in the alleviation of MDM2-dependent inhibition of p53 activity, whereas Ser 392 stimulates the DNA-binding activity of p53 (Kapoor et al. 2000).

HER2/neu encodes a 185 kD protein which is a member of the membrane-spanning type I receptor tyrosine kinase family (Harari and Yarden 2000). Overexpression of HER2/neu has been found in many types of cancers with high

frequency, suggesting its critical role in the development of human tumors (Hung and Lau 1999). Ectopic overexpression of HER2/neu to the high levels observed in some tumors is reported to enhance tumorigenicity in model systems (Di Fiore et al. 1987; Hudziak et al. 1988). HER2/neu is known to activate several signaling pathways, including mitogen-activated protein kinases (MAPK) (Ben Levy et al. 1994) and Phosphatidylinositol-3'-OH kinase (PI3-K) pathways (Peles et al. 1992). Both of these pathways are known to enhance cell proliferation and survival, although MAPK, when activated by some conditions of stress, may mediate apoptosis (Wang et al. 2000).

Therapy of human cancer often includes treatment with cisplatin-based combination regimens (Schiller 2001; du Bois 2001). Although Cisplatin has clinical utility against several tumor types, the presence of primary or the emergence of secondary resistance significantly undermines the curative potential of this drug (Siddik et al. 1999). In view of the central problem of cisplatin resistance, efforts have focused on the development of alternative platinum-based analogues. We have reported the compound 1R,2R-diaminocyclohexane-diacetato-dichloro-platinum (IV) (DACH-acetato-Pt; Figure 1) as a candidate with clinical potential in cisplatin resistance (Kido et al. 1993; Al-Baker et al. 1994) and mechanistic studies have been in progress to rationalize its activity.

Although the role of functional p53 on the sensitivity to cisplatin is still controversial (Fan et al. 1995; Hawkins et al. 1996), it is widely established that cisplatin-induced DNA damage activates signaling pathways culminating in p53 induction (Siddik et al. 1998). Recent reports have shown that the activation of extracellular signal-regulated protein kinase (ERK), which is a member of the MAPK, targets p53

phosphorylation at Ser 15 (Persons et al. 2000) and is required for cisplatin-induced apoptosis (Wang et al. 2000). DACH-acetato-Pt, on the other hand, has shown greater potency against cisplatin-resistant ovarian tumor cells with wild-type p53, but was less cytotoxic against cells having mutant or null p53 (Hagopian et al. 1999). This compound is very efficient in inducing p53 in cisplatin-resistant wild-type p53 ovarian tumor models, and disruption of wild-type p53 function increased resistance to the compound. These facts suggest that the potent antitumor activity of DACH-acetato-Pt is p53-dependent and that different signaling pathways are activated with this platinum agent than with cisplatin.

HER2/neu is also known to induce resistance to some antitumor agents and sensitivity to others (Andersen and Borresen 1995; Pegram et al. 1997). Although the mechanisms of drug resistance induced by HER2/neu are still unclear, there are some reports that suggest an interaction with pathways for p53 and p21 (Bacus et al. 1996; Yu et al. 1998). Recently, Casalini et al. (2001) reported that overexpression of HER2/neu promoted growth inhibition and apoptosis in tumor cells bearing wild-type p53 but was associated with proliferation in cells with mutant p53. It is also reported that ras-mediated signal transduction pathway, which is one of the major down-stream targets of HER2/neu signal, inhibits p53 function (Ries et al. 2000). This pathway is known to play a major role in the expression of resistance to DNA-damaging agents (Dempke et al. 2000). However, the effect of HER2/neu overexpression on p53 induction in response to DNA damage is yet to be clarified.

The purpose of this study is to assess the effects of HER2/neu overexpression on the sensitivity to cisplatin and the novel analog DACH-acetato-Pt in isogenic tumor

models with different p53 status. We report herein that increased levels of HER2/neu increased resistance to cisplatin only in cells bearing wild-type p53 but not mutant p53, whereas they increased sensitivity to DACH-acetato-Pt regardless of p53 status. In addition, overexpressed HER2/neu down-regulated p53 by suppressing its phosphorylation at Ser 15 and Ser 392 in cells with wild-type p53 following cisplatin treatment, but did not reduce p53 induction in response to DACH-acetato-Pt.

RESULTS

Status of HER2/neu in MCF-7 and MDA-MB-435 Cells. We have examined the status of HER2/neu in MCF-7 and MDA-MB-435 cell lines. As shown in Figure 2A, the low expression of HER2/neu is apparent in parental and neo cells of both models, whereas MCF-7/HER2-18 and MDA-MB-435/eB1 express substantially high levels of $p185^{HER2/neu}$. The SK-Br3 cell line, with established amplification and overexpression of HER2/neu gene, was used as a positive control. The levels of phophorylated $p185^{HER2/neu}$, p53 and related proteins are shown in Figure 2B. The active, phosphorylated form of $p185^{HER2/neu}$ was substantially higher in HER2/neu transfected cells than in neo-control cells. Interestingly, the basal expression of MDM2 was 1.4-fold higher in MCF-7/HER2-18 cells than that in neo-control cells. On the other hand, basal levels of p53 and p21 were similar in MCF-7/HER2-18 compared to neo, while a modest up-regulation of p21 was observed in MDA-MB-435/eB1 cells, as reported previously (Yu et al. 1998).

Cytotoxicity of Cisplatin and DACH-acetato-Pt. The results of cytotoxic evaluation are shown in Table 1. Increased levels of $p185^{HER2/neu}$ led to an increase in resistance to cisplatin about 2- to 3-fold in wild-type p53 MCF-7 model (IC_{50}'s, 0.34 vs. 0.94 μM for continuous drug exposure and 9.75 vs. 18.2 μM for 2-h exposure, respectively). In contrast, MCF-7/ HER2-18 cells demonstrated significant sensitivity to DACH-acetato-Pt by up to 3-fold compared to neo (IC_{50}'s, 0.055 vs. 0.18 μM for continuous drug exposure and 15.0 vs. 22.1 μM for 2-h exposure, respectively). Although the HER2/neu status did not significantly affect the sensitivity to cisplatin in mutant p53 MDA-MB-435 cells, the cytotoxicity of DACH-acetato-Pt was enhanced in

MDA-MB-435/eB1 cells compared to neo (IC_{50}'s, 1.31 vs. 1.75 μM for continuous drug exposure and 39.9 vs. 68.9 μM for 2-h exposure, respectively). On the other hand, IC_{50} of DACH-acetato-Pt was much higher in mutant p53 MDA-MB-435 cell lines than in wild-type p53 MCF-7 cell lines, suggesting p53-dependent cytotoxicity of this drug, as shown in our previous study (Hagopian et al. 1999).

Biochmical Pharmacology of Cisplatin and DACH-acetato-Pt. The results of biochemical pharmacology are shown in Table 2. Increased levels of $p185^{HER2/neu}$ affected neither cellular platinum uptake nor DNA adduct formation after 2-h exposure to cisplatin or DACH-acetato-Pt in both models. DNA damage tolerance is defined as the level of adducts that are required to kill 50% of the tumor cells. DNA damage tolerance to cisplatin was significantly higher in the MCF-7/HER2-18 than in neo (0.63 vs. 0.20 ng Pt/mg DNA), whereas there was no significant difference in damage tolerance between MDA-MB-435 cells exposed to cisplatin (0.93 vs. 1.1 ng Pt/mg DNA). On the other hand, the increased sensitivity to DACH-acetato-Pt was accompanied by a significant decrease in DNA damage tolerance both in MCF-7 (0.010 vs. 0.027 ng Pt/mg DNA) and in MDA-MB-435 (0.28 vs. 0.38 ng Pt/mg DNA) models. These results suggest that the modulation of cytotoxicity by HER2/neu overexpression on the sensitivity to the platinum agents was due entirely to the change in tolerance to platinum adducts.

Concentration-dependent Induction of Total and Phosphorylated p53 and Transactivation of p21 by Cisplatin and DACH-acetato-Pt. The cytotoxicity and biochemical pharmacology data shown above strongly suggest that the decrease in cisplatin sensitivity by HER2/neu overexpression is p53-dependent, whereas the increased sensitivity to DACH-acetato-Pt by HER2/neu is independent of p53. To

examine the role of HER2/neu on the activation of wild-type p53 in response to platinum-induced DNA damage, MCF-7 transfectants were exposed to cisplatin or DACH-acetato-Pt and cellular extracts subjected to Western analysis. Figure 3 shows the dose-dependent induction of p53 and related proteins following exposure to each drug. For both drugs, induction of total p53 is seen to be dependent on concentration both in MCF-7/neo and MCF-7/HER2-18. Although the phosphorylation of p53 at Ser 15 and Ser 392 is apparent with cisplatin treatment, a lower extent of Ser15 and a relatively poor phosphorylation at Ser 392 are observed with DACH-acetato-Pt. Increased levels of $p185^{HER2/neu}$ attenuated phosphorylation of p53 at both Ser 15 and Ser 392 with cisplatin, whereas no difference was apparent between both cell types with DACH-acetato-Pt. The transactivation of p21 is also seen to be dependent on the concentration of cisplatin in both cell types, and the extent is consistent with the levels of phosphorylated p53. In contrast, there is no difference in the transactivation of p21 between cells exposed to DACH-acetato-Pt. The expression of HER2/neu and the active phosphorylated form of $p185^{HER2/neu}$ were not affected by cisplatin or DACH-acetato-Pt at any concentration (data not shown).

Time-dependent Induction of p53 and Associated Proteins. The alterations of protein expression with time following a 2-h exposure to 20 μM drug concentration are shown in Figure 4. Levels of p53 and associated proteins increased with time following drug exposure. The results of densitometric analysis on time-course induction of p53 and p21 are shown in Figure 5. The levels of p53 induced by cisplatin peaked at 36 hr in control MCF-7 cells, and were greatly reduced by HER2/neu overexpression from 24 to 48 hr. The induction kinetics of p53 in response to DACH-acetato-Pt was

different than that with cisplatin. By 6 hr, DACH-acetato-Pt had induced p53 in MCF-7/neo cells to levels that approached peak levels observed with cisplatin at 24 hr. However, in the case of DACH-acetato-Pt, a reduction in p53 was not observed by HER2/neu overexpression. Instead, this overexpression appeared to sustain the induction of p53 beyond the 12hr time point. The temporal aspect of transactivation of p21 was consistent with the levels of p53 with both drugs. On the other hand, MDM2 was also transactivated in a p53-dependent manner (Figure 4), but unlike p21, the levels of MDM2 increased more rapidly in MCF-7/HER2-18 cells than in neo cells in response to both drugs. These results suggest that the up-regulation of MDM2 in HER2/neu–transfected cells is promoted by the overexpression of HER2/neu. The extent of phosphorylated p53 at Ser 15 and Ser 392 is shown in Figure 6. Increased levels of HER2/neu significantly suppressed the phosphorylation of p53 at both sites in response to cisplatin-induced DNA-damage, whereas reduced or poor phosphorylations at these p53 sites were seen in both cell types with DACH-acetato-Pt. It is likely that the suppression of p53 induction in MCF-7/ HER2-18 exposed to cisplatin was due to the decreased phosphorylations at the sites.

DISCUSSION

Human tumors rarely possess a single genetic defect, and it is likely that therapeutic outcome following chemotherapy will depend on the relative modulatory effect on each of the molecular targets. In our previous study, we have demonstrated that DACH-acetato-Pt is effective against refractory cancers possessing wild-type p53 (Hagopian et al. 1999). Since such tumors can also demonstrate amplification/ overexpression of HER2/neu (Thor et al. 1998), it was important to examine if this molecular defect could influence the potential therapeutic benefit of the platinum analog. On the other hand, several groups have reported that combination of gene therapy using functional p53 with DNA damaging agents may have a synergistic effect without additional toxicity (Fujiwara et al. 1994; Osaki et al. 2000). Consequently, several clinical studies are in progress and platinum drugs have been used as candidates for the DNA damaging agents (Roth et al. 2002). In order to evaluate the possibility of future gene-based therapy against HER2/neu-overexpressing tumors, it is important to also clarify the interaction of p53 and HER2/neu following platinum-induced DNA damage. In this study, we have demonstrated that, unlike cisplatin, the cytotoxicity of DACH-acetato-Pt is increased by HER2/neu overexpression.

The absence of functional p53 in tumor cells is known to be associated with resistance to chemotherapeutic agents (Lowe et al. 1993; 1994). As for cisplatin, however, the implication of functional p53 is unclear. Several studies have shown evidence for positive effects of functional p53 on cisplatin sensitivity. Gallagher et al. (1997) reported that expression of p53 genetic suppressor element decreased p53 protein levels resulting in an 8-fold increase in resistance to cisplatin in A2780 ovarian

carcinoma cells. Similarly, Gurnani et al. (1999) introduced adenovirus-mediated wild-type p53 into human cell lines and demonstrated significant increase in cisplatin sensitivity. On the contrary, several groups have reported that inactivation of p53 function with introduction of papilloma virus E6 gene enhanced sensitivity to cisplatin (Fan et al. 1995; Hawkins et al. 1996). Although the role of functional p53 on the sensitivity to cisplatin may depend on cell types, it is well established that cisplatin activates pathways for p53 induction (Siddik et al. 1998; Lakin and Jackson 1999).

One of the recent topics of interest on the regulation of p53 is the post-translational activation of this molecule in response to DNA damage (Albrechtsen et al. 1999; Lakin and Jackson 1999). Phosphorylation and acetylation at several sites have been reported to activate p53 protein. Among these modification sites, Persons et al. (2000) demonstrated that cisplatin-induced DNA damage resulted in phosphorylattion of p53 at Ser15 through activation of ERK-1, 2/MAPK pathway, which appeared to be required in cisplatin-induced apoptosis (Wang et al. 2000). In the present study, we have shown that cisplatin induced phosphorylation of p53 not only at Ser 15 but also at Ser 392. Kapoor et al. (2000) have also reported phosphorylation at both sites in response to UV radiation. They demonstrated that phosphorylation at Ser 15 was implicated in stabilization of p53, while phosphorylation at Ser392 (Ser 389 in mouse) increased its DNA-binding activity. Thus, cooperative phosphorylation at these sites was reported to activate p53 effectively (Kapoor et al. 2000). Consistent with the results from UV radiation, our results revealed that phosphorylation of these two sites contributed to both the stabilization and increased transactivational activity of p53 in response to cisplatin DNA damage. On the other hand, a lesser extent of Ser15 phosphorylation and a very

poor phosphorylation at Ser 392 were observed with DACH-acetato-Pt, suggesting that different mechanism of post-translational modifications is involved in the activation of p53 in response to DACH-acetato-Pt. This pattern of p53 modification is analogous to that reported by ionizing radiation (Kapoor and Lozano 1998), and may be critical in the effectiveness of the analog against cisplatin resistant tumor cells.

A significant body of evidence on chemoresistance induced by HER2/neu has been reported in experimental studies as well as in clinical studies (Harari and Yarden 2000; Pegram et al. 1997). In this study, we have shown that increased levels of HER2/neu led to a 2- to 3-fold increase in cisplatin resistance in wild-type p53 MCF-7 model, whereas it did not affect the sensitivity to cisplatin in mutant p53 MDA-MB-435 model. Pegram et al. (1997) have reported the effect of HER2/neu overexpression on chemotherapeutic drug sensitivity in human breast and ovarian cancer cells. Although the authors did not focus on p53 status, overexpression of HER2/neu significantly decreased the sensitivity to cisplatin of their two tumor cell lines with wild-type p53, including MCF-7. In contrast, there was no significant difference in sensitivity to cisplatin in 3 out of the 4 cell lines with mutant p53, including MDA-MB-435. These findings are consistent with our results, suggesting that HER2/neu-induced resistance to cisplatin is p53-dependent. On the other hand, HER2/neu-transfected cells showed significant sensitivity to the novel platinum analog DACH-acetato-Pt regardless of p53 status. In addition, we have also shown that the modulation of cytotoxicity by HER2/neu was due entirely to the change in tolerance to platinum-induced DNA damage. These data suggest that overexpression of HER2/neu results in greater tolerance to DNA-platinum adducts through down-regulation of signaling pathways to p53 when cisplatin is

the DNA damaging agent, whereas it increased the sensitivity to DACH-acetato-Pt through a reduction in adduct tolerance, which was independent of the p53 status.

An important fact we have uncovered in the present study is that increased levels of HER2/neu attenuate the phosphorylation of p53 both at Ser15 and Ser392 following cisplatin-induced DNA damage in wild-type p53 cell lines. As a result, both p53 induction and the p21 product from its transcriptional activity were significantly suppressed in MCF7/HER2-18 cells compared to control cells. Although the precise mechanism of how HER2/neu down-regulates p53 remains unclear, an explanation to account for this may be realized by considering the upregulation of MDM2 levels observed in MCF-7/HER2-18 cells. MDM2 binds p53 close to its N-terminus and acts as an ubiquitin ligase (Honda et al. 1997; Lakin and Jackson 1999), and phosphorylation of p53 at Ser 15 is known to inhibit binding of MDM2 to p53 and blocks MDM2-mediated degradation of p53 (Shieh et al. 1997). Recently, Zhou et al. (2001) reported that HER2/neu induced MDM2 phosphorylation at Ser 166 and Ser 186 through PI3-K pathways. According to their model, these phosphorylations of MDM2 enhance its nuclear localization, which increases p53 ubiquitination via its interaction with the transcriptional co-activator CPB/p300. This model strongly supports our findings with cisplatin.

In contrast, increased levels of HER2/neu did not reduce the induction of p53 in response to DACH-acetato-Pt. Instead, overexpression of HER2/neu sustained the induction of p53 after the 12hr time point, suggesting a positive effect of $p185^{HER2/neu}$ signaling on the sensitivity to DACH-aceato-Pt. Moreover, HER2/neu enhanced the cytotoxicity of DACH-acetato-Pt regardless of p53 status, although DACH-acetato-Pt

was much more potent against wild-type p53 MCF-7 cells than against mutant p53 MDA-MB-435 cells. These results, demonstrating a dependency on p53 status for potency, are consistent with our previous report using ovarian tumor models (Hagopian et al. 1999). These findings indicate that introduction of functional p53 in combination with DACH-acetato-Pt may be an effective treatment against cells lacking functional p53 and overexpressing HER2/neu.

In this study, we have demonstrated that overexpression of HER2/neu leads to cisplatin resistance by down-regulating p53 in wild-type p53 cell lines, but it dose not affect the sensitivity in mutant p53 cell lines. HER2/neu overexpression, on the other hand, increases the sensitivity to DACH-acetato-Pt independent of p53. These results suggest that cisplatin and DACH-acetato-Pt activate independent signal transduction pathways that regulate p53, and that HER2/neu impinges only on the pathway activated by cisplatin. Therefore, we postulate that DACH-acetato-Pt may have clinical utility in the management of tumors overexpressing HER2/neu, particularly against a wild-type p53 background from potency consideration.

MATERIALS AND METHODS

Chemicals. Cisplatin was obtained from Sigma Chemical Co. (St. Louis, MO). We have previously reported the synthesis and chemical characterization of DACH-acetato-Pt (Al-Baker et al. 1994). Cisplatin and DACH-acetato-Pt were dissolved in normal saline and water, respectively, then sterilized through 0.22-μm disc filters. The concentration of each drug was confirmed by flameless atomic absorption spectroscopy (FAAS) (Siddik et al. 1987). MTT was purchased from Sigma Chemical Co. (St. Louis, MO).

Cell Lines. MCF-7/HER2-18 having wild-type p53 and MDA-MB-435/eB1 with mutant p53, both of which are stably transfected with full-length HER2/neu cDNA, and control isogenic neo cell lines have been described previously (Yu et al. 1998; Benz et al. 1993). The cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine and antibiotics under a humidified atmosphere of 5 % CO_2.

Cytotoxicity and Biochemical Pharmacology Studies. For cytotoxic determinations, 500 to 1000 cells were plated in 100 μl of medium in 96-well plates. Following 2 days of incubation, cells were exposed to various concentrations of cisplatin or DACH-acetato-Pt. After another 4 or 5 days, the relative sensitivities of the cells to the platinum complexes were evaluated using a modified MTT assay (Carmichael et al. 1987). Evaluations in attached cells of cellular platinum uptake and DNA adduct formation were conducted as described previously (Kido et al. 1993; Yoshida et al. 1994). Briefly, cells treated with cisplatin or DACH-acetato-Pt (100 or 200 μM) for 2 h at 37 °C were microfuged and washed. For determination of cellular uptake, aliquots of

cell pellets were digested overnight at 55 °C in 50 μl of 1 M hyamine hydroxide (ICN, Irvine, CA). To measure platinum-DNA adduct formation, high molecular weight DNA was isolated from cell pellets according to standard procedures (Maniatis et al. 1982). The platinum content of samples was determined by FAAS. Platinum-DNA damage tolerance was defined as the value of DNA adducts at an IC_{50} concentration (Johnson et al. 1997).

Western Analysis Cells were exposed for 2 h to various concentration of cisplatin or DACH-acetato-Pt, washed, and incubated at 37 °C in drug-free medium for indicated time. For dose-dependent analysis, the cells were harvested after 24 h incubation. For time-course study, cells were exposed to 20 μM of each drug. The cells were then washed twice with ice-cold phosphate-buffered saline and lysed for 20 min on ice with 100 μl of lysis buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.02% sodium azide, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate, 100 μg/ml phenylmethylsulfonyl fluoride, and 1 μg/ml aprotinin). The lysates were collected by microcentrifugation at 4 °C, and then the protein was determined by the standard Lowry procedure. Forty μg of total cell protein was electrophoresed on a 7.5% (for HER2/neu) or 10% (for p53, p21, MDM2) SDS-polyacrylamide gel, transferred onto nitrocellulose membranes, and incubated with various antibodies. Mouse monoclonal anti-p53 (DO-1), anti- MDM2 (Ab-1) and anti-HER2/neu (c-neu, Ab-3) antibodies were obtained from Oncogene Research Products (Cambridge, MA), and anti-p21 (Cip1/Waf1) antibody was purchased from Transduction Laboratories (Lexington, KY). Rabbit polyclonal anti-phospho-HER2/neu (Y1248) antibody was obtained from Upstate Biotechnology (Lake Placid, NY) and Rabbit polyclonal anti-phospho-p53 antibodies (Ser 15 and Ser 392)

were purchased from Cell Signaling Technology (Beverly, MA). Mouse monoclonal anti-β-actin antibody was purchased from Sigma (St. Louis, MO). All immunoblots were visualized by enhanced chemiluminescence detection system (Amersham, Arlington Heights, IL), and quantified by laser densitometry.

Statistical Analysis. Differences between groups were evaluated by paired Student's *t* test in the cytotoxicity assays and the biochemical pharmacology and by unpaired *t* test in the DNA-damage tolerance, respectively.

ACKNOWLEDGEMENTS

This work was supported by the U.S. Army Grant DAMD 17-99-1-9269, and in part by NCI RO1 CA77332 and RO1 CA82361 to Zahid H. Siddik.

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FIGURE LEGENDS

Figure 1. Structures of cisplatin and DACH-acetato-Pt (IV).

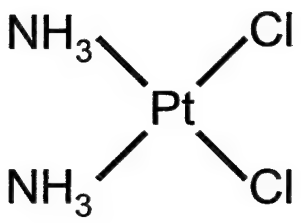
Figure 2. A. Western immunoblot of basal levels of $p185^{HER2/neu}$ in MCF-7 and MDA-MB-435 transfection models. SK-Br3 cells were used as a positive control. B. Basal evpression levels of phosphorylated $p185^{HER2/neu}$, p53 and related proteins.

Figure 3. Concentration-dependent induction of total and phosphorylated p53 and transactivation of p21 in MCF-7 cell lines exposed to cisplatin or DACH-acetato-Pt. Cells were exposed to various concentrations of drugs for 2h and then incubated in drug-free medium. Cells were harvested 24 h later, and protein was extracted and sujected to Western analysis.

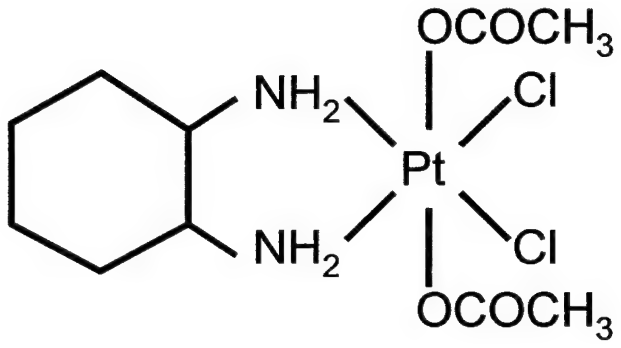
Figure 4. Time-dependent induction of p53 and associated proteins in MCF-7 models after treatment with cisplatin or DACH-acetato-Pt. Cells were treated with 20 μM of drugs for 2-h and subsequently incubated without drug for indicated time. Cells were harvested, and protein was extracted and examined for p53 and associated proteins by Western analysis.

Figure 5. Levels of p53 and p21 during the time-course estimated from immunoblots by laser densitometry.

Figure 6. Levels of phosphorylated p53 at Ser 15 and Ser 392 during the time course estimated from immunoblots by laser densitometry.



Cisplatin



1R,2R-DACH-$(Ac)_2Cl_2$-Pt(IV)

Figure 1

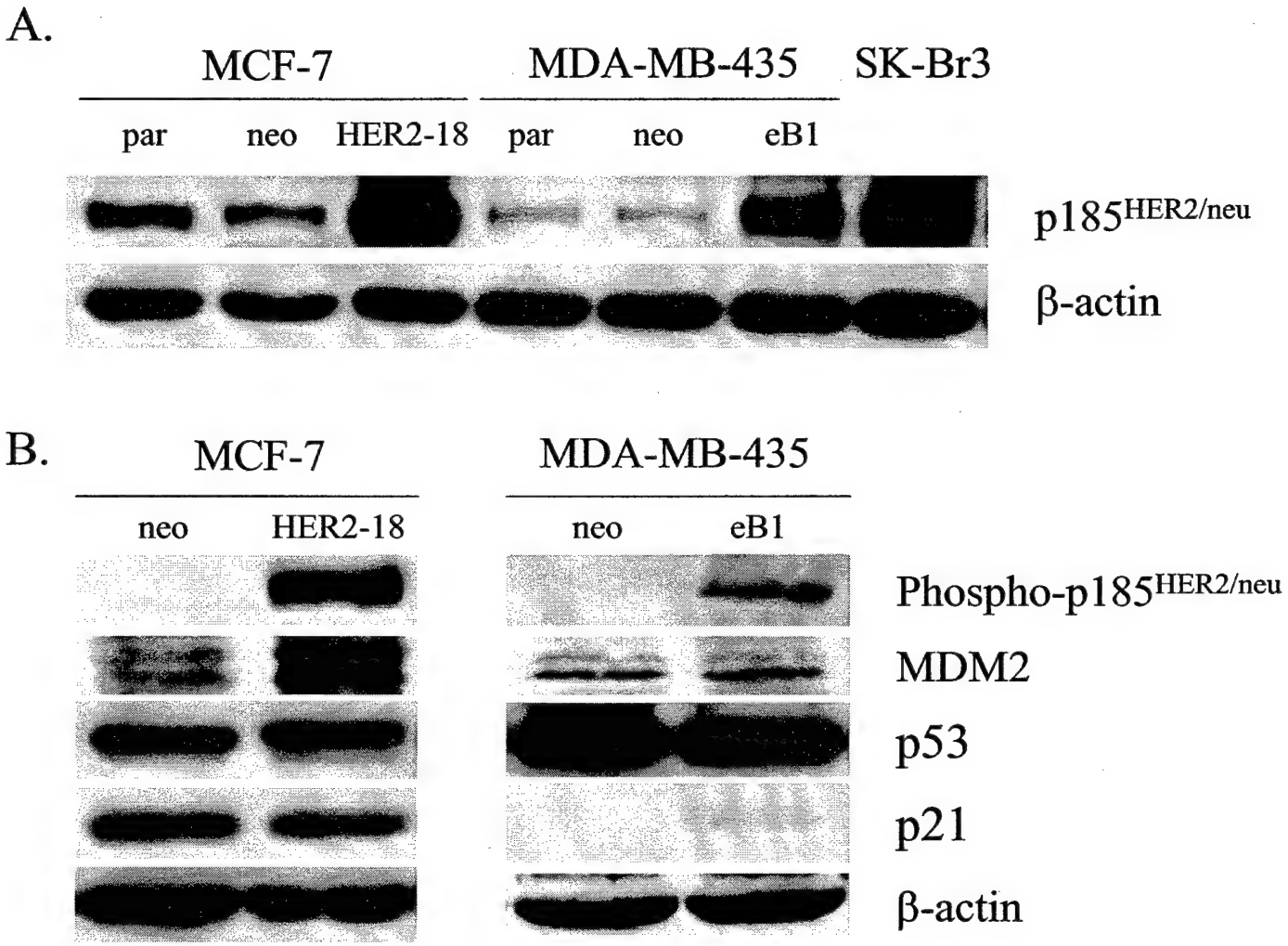
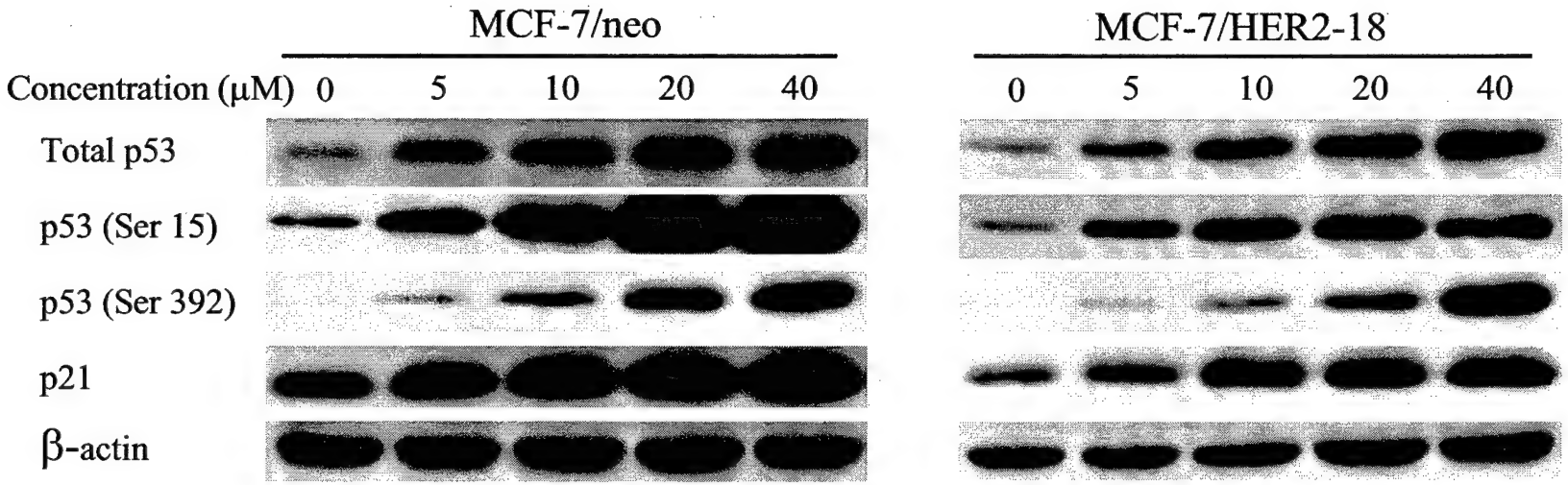

A.
MCF-7
MDA-MB-435
SK-Br3
par
neo
HER2-18
par
neo
eB1
p185HER2/neu
β-actin
B.
MCF-7
MDA-MB-435
neo
HER2-18
neo
eB1
Phospho-p185HER2/neu
MDM2
p53
p21
β-actin

Figure 2

A. Cisplatin



B. DACH-acetato-Pt

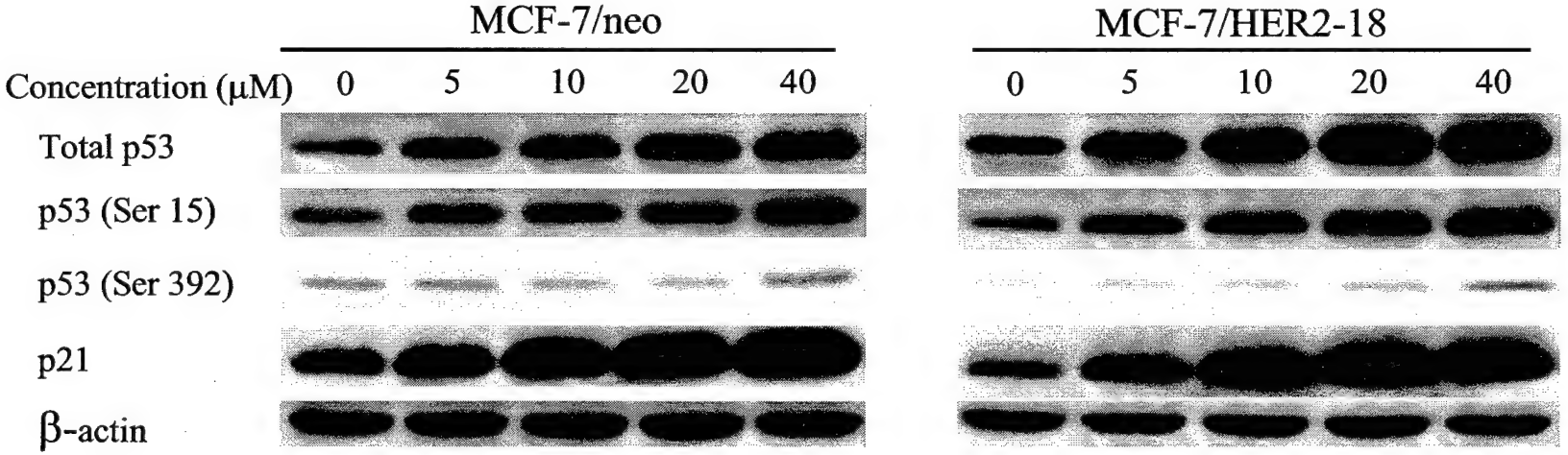
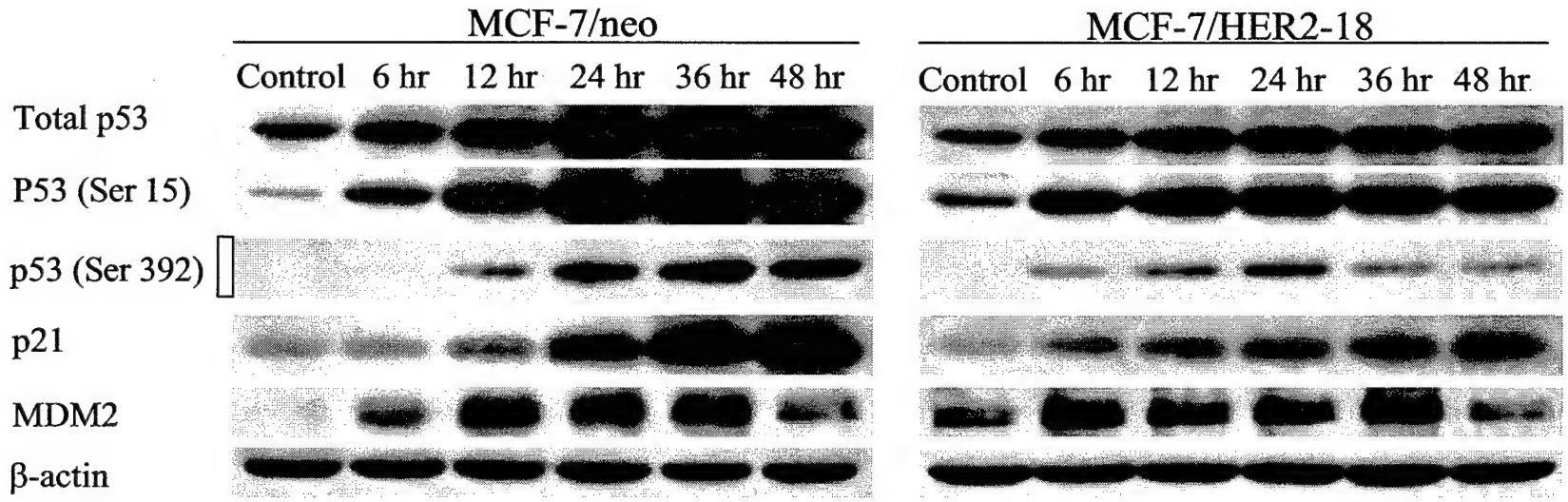


Figure 3

A. Cisplatin



B. DACH-acetato-Pt

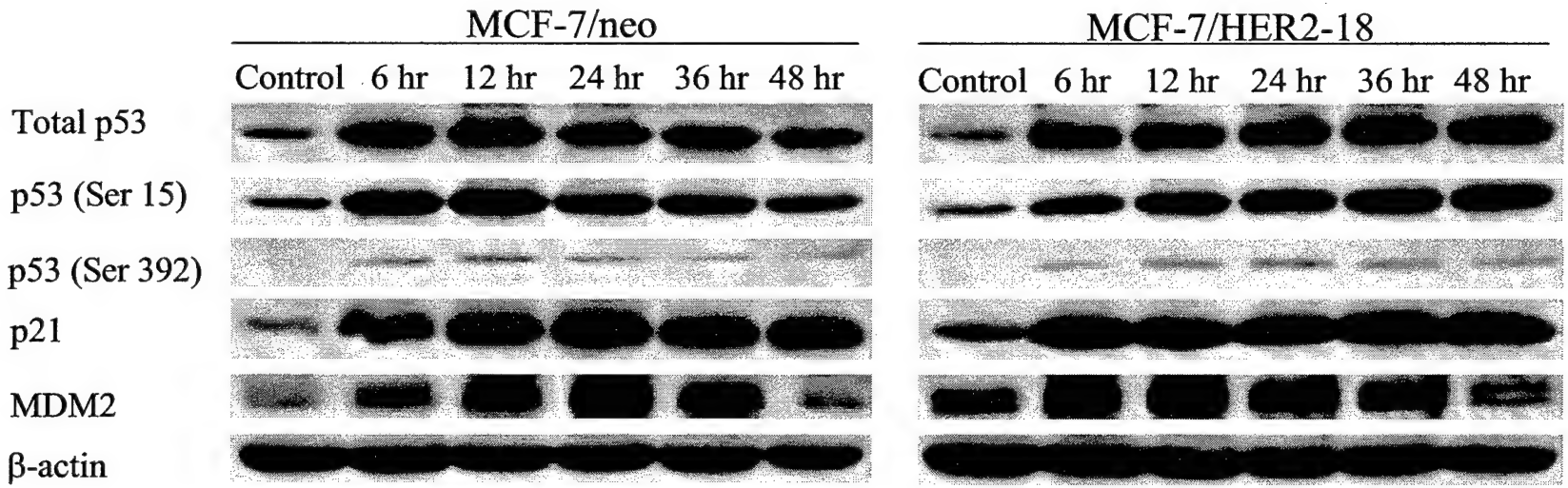


Figure 4

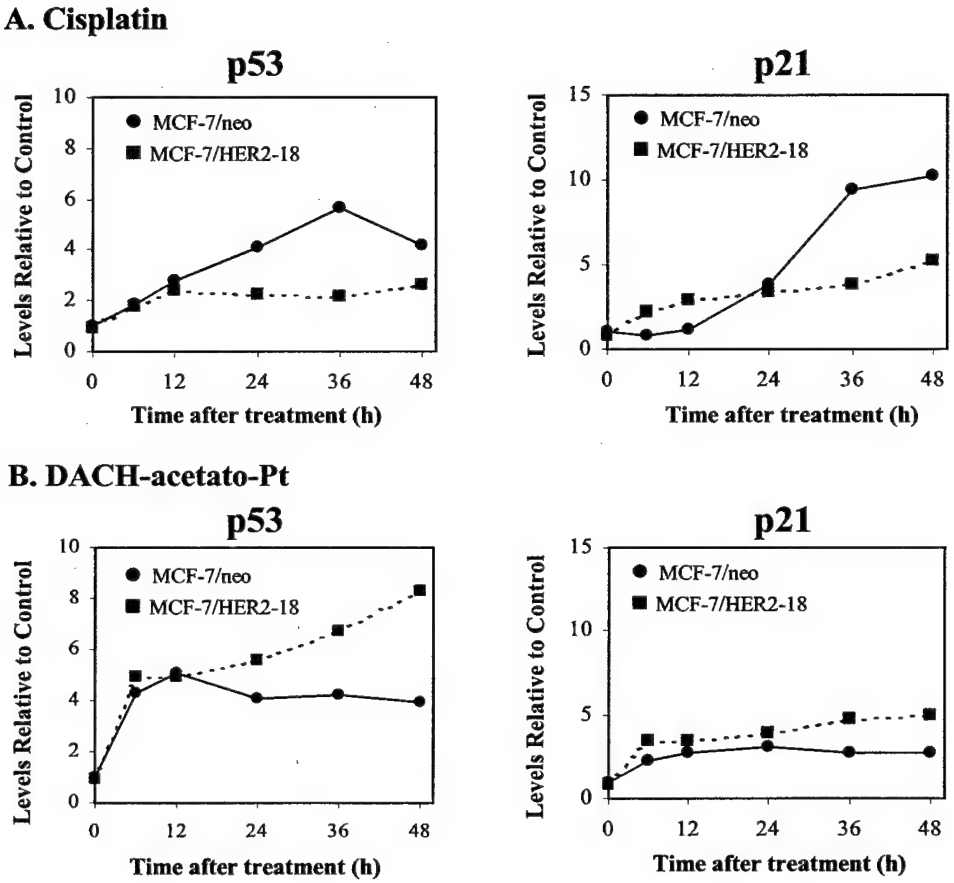
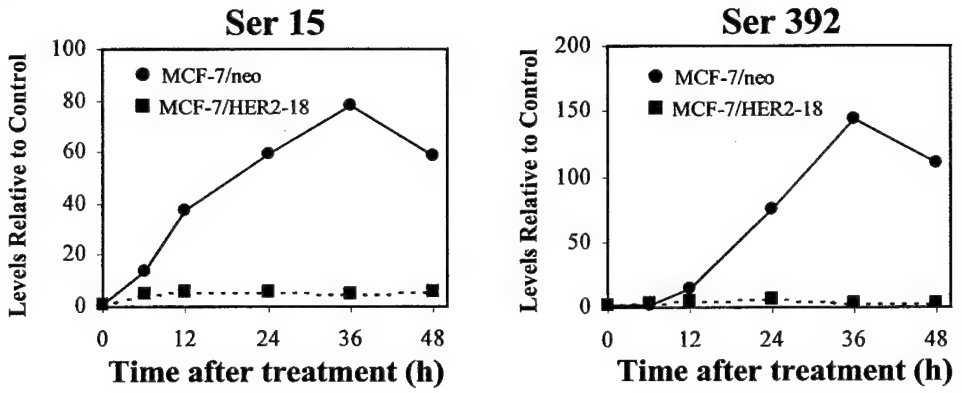

A. Cisplatin
p53
p21
MCF-7/neo
MCF-7/HER2-18
Levels Relative to Control
Time after treatment (h)
B. DACH-acetato-Pt
p53
p21
MCF-7/neo
MCF-7/HER2-18
Levels Relative to Control
Time after treatment (h)

Figure 5

A. Cisplatin



B. DACH-acetato-Pt

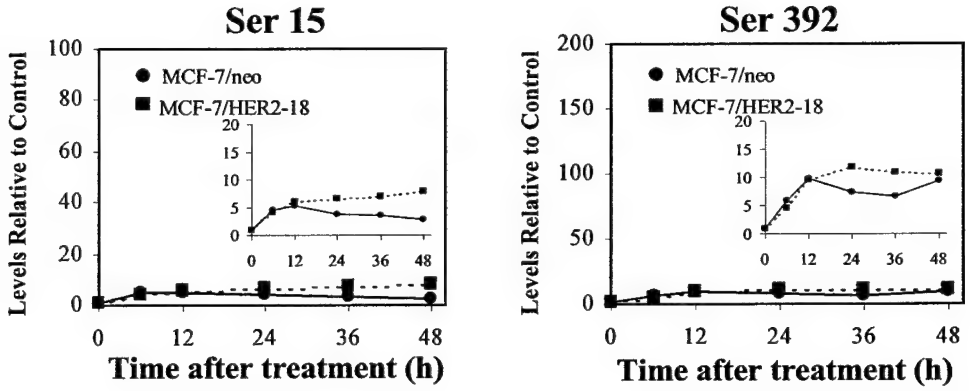


Figure 6

Table 1. Effect of HER2/neu overexpression on the cytotoxicity of cisplatin and DACH-acetato-Pt following continuous or 2-h drug exposure

	Continuous drug exposure		2-h drug exposure	
Cell line	Cisplatin	DACH-acetato-Pt	Cisplatin	DACH-acetato-Pt
MCF-7/neo	0.34 ± 0.04*	0.18 ± 0.08	9.75 ± 1.73	22.1 ± 4.0
MCF-7/HER2-18	0.94 ± 0.31**	0.055 ± 0.016**	18.2 ± 4.9**	15.0 ± 3.3**
MDA-MB-435/neo	1.41 ± 0.33	1.75 ± 0.41	5.63 ± 0.52	68.9 ± 8.2
MDA-MB-435/eB1	1.15 ± 0.12	1.31 ± 0.34**	4.99 ± 0.30	39.9 ± 1.0**

*Mean ± SD; **$P<0.05$, vs. neo with paired t-test; n=3-5

Table 2. Biochemical pharmacology of cisplatin and DACH-acetato-Pt against MCF-7 and MDA-MB-435 transfectants

	Cellular uptake (ng platinum/mg protein)		DNA adduct formation (ng platinum/mg DNA)		DNA-damage tolerance (ng platinum/mg DNA)	
Cell line	Cisplatin	DACH-acetato-Pt	Cisplatin	DACH-acetato-Pt	Cisplatin	DACH-acetato-Pt
MCF-7/neo	145.4 ± 21.4*	55.6 ± 9.1	59.8 ± 17.7	15.1 ± 4.5	0.20 ± 0.03	0.027 ± 0.012
MCF-7/HER2-18	149.9 ± 42.5	71.7 ± 17.6	66.5 ± 13.8	17.9 ± 3.9	0.63 ± 0.21**	0.010 ± 0.003**
MDA-MB-435/neo	217.4 ± 28.0	142.1 ± 38.4	75.2 ± 13.5	21.5 ± 5.0	1.1 ± 0.3	0.38 ± 0.09
MDA-MB-435/eB1	266.7 ± 87.2	120.1 ± 37.9	81.2 ± 7.7	21.1 ± 5.4	0.93 ± 0.10	0.28 ± 0.07**

*Mean ± SD; **P<0.05, vs. neo with unpaired *t*-test; n=3-6

APPENDIX – MS #2 (in preparation)

A novel analog 1R,2R-Diaminocyclohexane-diacetato-dichloro-Pt (IV) circumvents cisplatin resistance induced by upregulation of $p21^{waf1/cip1}$ in breast cancer cell lines[1]

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Running Title: p21 and platinum resistance

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INTRODUCTION

Breast cancer is the most frequently diagnosed malignancy in American women, and the second most common cause of cancer death. In 2002, it was predicted that an estimated 203,500 new cases would be diagnosed of this disease and approximately 39,600 would die in the United States {Jemal, Thomas, et al. 2002 34 /id}. Although approximately 80% of breast cancer patients present with disease limited to the breast and/or axillary lymph nodes, almost half of these patients later develop metastatic disease and eventually succumb to it{Fornier, Munster, et al. 1999 35 /id}. Therefore, systemic therapy, including chemotherapy and hormonal manipulation, is in a great position for the management of breast cancer patients, especially with advanced or metastatic diseases, while surgical and radiotherapeutic interventions contribute to local control.

Cisplatin is among the most widely used and broadly active antitumor drugs {du 2001 54 /id}{Schiller 2001 53 /id}. Despite its wide spectrum of clinical activity, however, cisplatin has not been used for the treatment of breast cancer patients. One of the major reasons is that early clinical studies with heavily pretreated patients demonstrated little or no activity {Yap, Salem, et al. 1978 38 /id}. On the contrary, several groups have later revealed that the overall response rate among patients with advanced breast cancer given high-dose cisplatin without prior chemotherapy was 42 –54 % {Sledge, Loehrer, et al. 1988 40 /id}{Kolaric & Roth 1983 39 /id}, suggesting that cisplatin may be an effective agent against this disease. More recently, several phase studies using cisplatin combined with anthracyclines {Nielsen, Dombernowsky, et al. 2000 41 /id}or taxanes {Rosati, Riccardi, et al. 2000 42 /id} have indicated a very high potential of cisplatin in the combination setting for the treatment of patients with

advanced or metastatic breast cancer. In spite of the increased evidence for the clinical utility of cisplatin for breast cancer patients, the response rates of the combination are not durable and do not translate into an improved 5-year survival rate. This is likely attributable to the development of drug resistance, which is a persuasive factor in the quest of novel agents.

The presence of primary or the emergence of acquired resistance to cisplatin is indeed a major limitation in medical oncology {Siddik, Hagopian, et al. 1999 18 /id}. Several mechanisms have been implicated for this, including decreased drug accumulation, increase in thiol levels and increased DNA repair {Timmer-Bosscha, Mulder, et al. 1992 36 /id}. In addition to these classical resistance mechanisms, there is considerable evidence to show that genetic alterations in tumors, including activation of oncogenes and inactivation of tumor suppressor genes, could modify the cisplatin-induced signaling pathways, and thereby increase the tolerance to DNA damage {Dempke, Voigt, et al. 2000 30 /id}. Since Burchenal et al. demonstrated that specific platinum analogs could circumvent cisplatin resistance, efforts have been made to identify alternative platinum analogs that are effective against resistant tumors {Burchenal, Kalaher, et al. 1979 38 /id}. In this regard, we have reported that the analog 1R,2R-diaminocyclohexane-diacetato-dichloro-platinum (IV) (DACH-acetato-Pt, Figure1) is a candidate with clinical potential in cisplatin resistance {Al-Baker, Siddik, et al. 1994 31 /id}.

DACH-acetato-Pt appears to have greater potency against cisplatin-resistant ovarian tumor cells with wild-type p53, but was less toxic against cells having mutant or null p53 {Hagopian, Mills, et al. 1999 45 /id}. Moreover, this compound is very efficient

in inducing p53 in cisplatin-resistant wild-type p53 tumor models, and disruption of p53 function increased resistance to the compound. We have proposed that DACH-acetato-Pt and cisplatin activate independent signaling pathways for p53 induction, and this characteristic may be significant in the potential management of cisplatin-resistant ovarian tumors, particularly against a wild-type p53 background {Hagopian, Mills, et al. 1999 45 /id}. Breast and ovarian cancers are known to develop similar genetic impediments, such as mutation/deletion of p53, amplification/overexpression of HER2/neu, loss of BRCA function {Welcsh & King 2001 37 /id}, and estrogen-dependency. As it is feasible that DACH-acetato-Pt may have similar modulatory effect on each molecular targets in both disease types, the novel platinum analog may have a potential against breast tumors in addition to that proposed for ovarian cancer.

In this study, we have compared the cytotoxicity of cisplatin and DACH-acetato-Pt in a panel of breast cancer cell lines and have examined the basis for the relative sensitivity of the tumor panel toward each agents. We report here that the basal levels of universal cyclin-dependent kinase (cdk) inhibitor $p21^{Waf1/Cip1}$ correlated with the resistance to cisplatin, while the sensitivity to DACH-acetato-Pt depended on the presence of functional p53 but was not affected by the upregulation of p21.

MATERIALS AND METHODS

Chemicals. Cisplatin was obtained from Sigma Chemical Co. (St. Louis, MO). We have previously reported the synthesis and chemical characterization of DACH-acetato-Pt {Al-Baker, Siddik, et al. 1994 31 /id}. Cisplatin and DACH-acetato-Pt were dissolved in normal saline and water, respectively, then sterilized through 0.22-μm disc filters. The concentration of each drug was confirmed by flameless atomic absorption spectroscopy (FAAS) {Siddik, Boxall, et al. 1987 32 /id}. MTT was purchased from Sigma Chemical Co. (St. Louis, MO).

Cell Lines. The nine human breast cancer cell lines used in this study were obtained from American Type Culture Collection (ATCC, Rockville, MD). Cells were grown as monolayers in 5% CO_2 and 95% humidified air at 37 °C. The MCF-7, HCC1937, MDA-MB-435, MDA-MB-436 and T47D cell lines were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine and antibiotics. The ZR75-1 and MDA-MB-157 cell lines were grown in Dulbecco's modified Eagle's medium, 10% heat-inactivated FBS, 2 mM L-glutamine, 10 μg/ml Insulin and antibiotics. An almost identical medium, containing 20% heat-inactivating FBS, was used to grow the MDA-MB-330 cell line. The SK-Br3 was maintained in McCoy's 5a medium supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine and antibiotics.

Analysis of p53 status by Wave analysis and DNA sequencing. Mutation of p53 in ZR75-1 and MDA-MB-330 cell lines was screened by the denaturing high-performance liquid chromatography (DHPLC) technique {Gross, Kiechle, et al. 2001 39 /id}, and suspected exons were subjected to DNA sequencing for confirmation of

mutation. Briefly, primer sets spanning exon 5 to exon 10 of p53 were designed and appropriate heteroduplex detection in DHPLC analysis was designed. DHPLC analysis was performed on a Wave DNA Fragment Analysis System (Transgenomic, San Jose, CA) as previously described {Gross, Arnold, et al. 1999 40 /id}. PCR products demonstrating heteroduplex formation were subjected to sequence analysis.

Cytotoxicity and Biochemical Pharmacology Studies. For cytotoxic determinations, 500 to 1000 cells were plated in 100 μl of medium in 96-well plates. Following a 24-hour incubation, cells were exposed continuously to various concentrations of cisplatin or DACH-acetato-Pt. After another 7 days for the MDA-MB-330 cell line and 5 days for the other cell lines, the relative sensitivities of the cells to the platinum complexes were evaluated using a modified MTT assay {Carmichael, DeGraff, et al. 1987 34 /id}. Evaluations in attached cells of cellular platinum uptake and DNA adduct formation were conducted as described previously {Yoshida, Khokhar, et al. 1994 35 /id}{Kido, Khokhar, et al. 1993 19 /id}. Briefly, cells treated with 100 μM cisplatin or DACH-acetato-Pt for 2 h at 37 °C were microfuged and washed. For determination of cellular uptake, aliquots of cell pellets were digested overnight at 55 °C in 50 μl of 1 M hyamine hydroxide (ICN, Irvine, CA). To measure platinum-DNA adduct formation, high molecular weight DNA was isolated from cell pellets according to standard procedures {Maniatis, Frisch, et al. 1982 36 /id}. The platinum content of samples was determined by FAAS. Platinum-DNA damage tolerance was defined as the level of DNA adducts formed at an IC_{50} concentration {Johnson, Laub, et al. 1997 37 /id}

Western Analysis Cells were exposed to various concentration of cisplatin or DACH-acetato-Pt for the indicated length of time. For dose-dependent analysis, the cells

were harvested after 24 h incubation. For time-course study, cells were exposed to 10 μM of each drug, washed twice with ice-cold phosphate-buffered saline (PBS), and lysed for 20 min on ice with 100 μl of lysis buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.02% sodium azide, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate, 100 μg/ml phenylmethylsulfonyl fluoride, and 1 μg/ml aprotinin). The lysates were collected by microcentrifugation at 4 °C, and the protein determined by the standard Lowry procedure. Forty μg of total cell protein was electrophoresed on a 10% SDS-polyacrylamide gel, transferred onto nitrocellulose membranes, and incubated with various antibodies. Mouse monoclonal anti-p53 (Ab-6, OP43) and anti-$p185^{HER2/neu}$ (Ab-3, OP15) antibodies, and Rabbit polyclonal anti-cyclin E (PC438) were obtained from Oncogene Research Products (Cambridge, MA), and anti-p21 (Cip1/Waf1, C24420) antibody was purchased from Transduction Laboratories (Lexington, KY). Rabbit polyclonal anti-cyclin D1 (sc-718) and anti-p27 (kip1, #06-455) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and Upstate Biotechnology (Lake Placid, NY), respectively. Mouse monoclonal anti-β-actin antibody was purchased from Sigma (St. Louis, MO). All immunoblots were visualized by enhanced chemiluminescence detection system (Amersham, Arlington Heights, IL), and quantified by laser densitometry.

Cell cycle analysis. Attached cells in an exponential growth phase in 100-mm tissue culture dishes were exposed for upto 48 h to various concentrations of cisplatin or DACH-acetato-Pt. The cells were collected, washed twice with ice-cold PBS, and fixed with a 1% final concentration of paraformaldehyde in PBS for 15 minutes on ice. The cells were then washed with ice-cold PBS, resuspended in 70% ethanol, and stored at -

20°C. Before analysis, the cell suspension was thawed, washed once with ice-cold PBS, resuspended in a solution of propidium iodide (10 μg/ml) in PBS containing 0.5% Tween 20 and 500 units/ml of RNase A (Sigma) and incubated at room temperature for 30 minutes and then at 4°C overnight. Cell cycle kinetics were determined on a Coulter Epics XL-MCL flow cytometer (Beckman Coulter Inc, Fullerton, CA) and analyzed by Multicycle Software (Phoenix Flow Systems, San Diego, CA).

RESUTLS

p53 status of breast cancer cell lines. p53 status of the nine breast cancer cell lines is shown in Table 1. There were two cell lines with wild-type p53, five with mutant p53 and two demonstrating no expression of p53. Among the mutant p53 cell lines, HCC1937 is unique in that it has mutation leading to termination at codon 306 in exon 8 {Tomlinson, Chen, et al. 1998 41 /id}, which resulted in a truncated p53 protein with an intact DNA binding domain and without any change in the amino acid sequence (Figure 2A). Although this type of mutant p53 is known to have 10 to 100 times lower affinity for DNA, such p53 variants are still able to bind DNA and stimulate transcription {Balagurumoorthy, Sakamoto, et al. 1995 42 /id}. In fact, the basal level of p21 in HCC1937 was similar to that seen in wild-type p53 MCF-7 cell line, and p21 was transactivated in response to both platinum agents in the HCC1937 cell line (data not shown). Based on the above evidence, the HCC1937 cells were grouped with cell lines having functional p53 in the following studies.

Cytotoxic evaluation of cisplatin and DACH-acetato-Pt in breast cancer cell lines. The results from the cytotoxic evaluation of platinum analogs in the panel of breast cancer cell lines are shown in Table 2. In order to evaluate the effect of wild-type p53 on the sensitivity to the platinum agents, the panel of cell lines was divided into two groups according to the status of functional p53. There was no difference in the median IC_{50} of cisplatin between the groups (~0.8 μM in bothe groups). On the other hand, the group with functional p53 demonstrated a lower median IC_{50} value for DACH-acetato-Pt than that without functional p53 (0.23 vs. 1.1-1.3 μM). The cisplatin/DACH-acetato-Pt potency ratio indicates a higher median value for the group possessing wild-type p53

function compared to that without this function (4.9 vs. 0.6-0.7). These results suggest that the potent cytotoxicity of DACH-acetato-Pt against breast cancer cell lines is also wild-type p53-dependent, as was shown in our previous study with the ovarian cancer panel {Hagopian, Mills, et al. 1999 45 /id}.

Biochemical pharmacology of cisplatin and DACH-acetato-Pt against ZR75-1 and T47D breast cancer cell lines. In our previous report, we demonstrated that an increase in DNA damage tolerance was a major mechanism of cisplatin resistance {Siddik, Mims, et al. 1998 52 /id}. In order to understand the biochemical pharmacologic basis of cisplatin resistance and sensitivity to DACH-acetato-Pt, particularly that of the mutant p53 T47D model, we have investigated drug uptake, DNA adducts and tolerance to DNA damage in wild-type p53 ZR75-1 and mutant p53 T47D cell lines. The results of the biochemical pharmacology study are shown in Table 3. The extent of platinum uptake was similar in both cell lines for each drug. DNA adducts in cells exposed to cisplatin were 1.5- to 2-fold higher than those in cells exposed to DACH-acetato-Pt. Thus the increased sensitivity of DACH-acetato-Pt relative to cisplatin was not due to either greater intracellular uptake or formation of the cytotoxic DNA adducts. The tolerance to cisplatin-induced DNA damage, on the other hand, was much higher than that induced by DACH-acetato-Pt in both cell lines, suggesting that relative potency of the two agents is linked to relative tolerance of the cell lines to DNA damage.

Basal expression levels of $p185^{HER2/neu}$, p53 and G1 cell cycle regulators. In order to determine the molecular factor which affects the cytotoxicity of cisplatin, basal expression levels of $p185^{HER2/neu}$, p53 and G1 cell cycle protein were investigated in the breast cancer cell lines (Figure 2B). Consistent with the literature, basal expression of

mutant p53 was much higher than p53 in the functionally-competent group. $p185^{HER2/neu}$ was highly overexpressed in MDA-MB-330 and SK-Br3 cell lines, moderately overexpressed in ZR75-1, HCC1937 and T47D cell lines and not overexpressed in the other four cell lines. Cells containing functional p53 (MCF-7, ZR75-1 and HCC1937) expressed higher basal levels of p21 when compared to cells without functional p53, as has been reported previously {El Deiry, Tokino, et al. 1995 44 /id} {El Deiry, Harper, et al. 1994 43 /id}. ZR75-1 expressed p21 levels that were about 3.5-fold higher than MCF-7, whereas HCC1937 cells exhibited a level that was 25% lower than in the MCF-7 model. Among the group of cell lines without functional p53, basal levels of p21 were only observed in MDA-MB-330, T47D and MDA-MB-157 cells, and relative expression levels compared to MCF-7 were 0.24-, 0.11-, and 0.05-fold, respectively. The other regulators of G1/S phase of the cell cycle (p27, Cyclin D1 and Cyclin E) were upregulated in unison in some cell lines, independent of p53 function. Note the multiple bands of Cyclin E, which has been reported previously {Gray-Bablin, Zalvide, et al. 1996 53 /id}.

Correlation between the cytotoxicity of cisplatin and relative expression levels of p21. We have examined the correlation between the markers examined in Figure 2B and IC_{50} values of the platinum analogs, and found that the basal expression levels of p21 loosely correlated with the relative sensitivity to in both functional p53-positive and -negative groups (Figure 3). In contrast, the correlation between relative p21 expression and IC_{50} values for DACH-acetato-Pt was not significant.

Induction of p53 and transactivation of p21 in breast cancer cell lines. In order to evaluate the role of p53 in response to the platinum drugs, cells were treated with

5 x IC_{50} concentrations of cisplatin or DACH-acetato-Pt for 24 h, and the cell extracts were subjected to Western immunoblot analysis (figure 4). The levels of p53 were increased in both wild-type p53 cell lines, whereas no significant inducion of p53 was seen in cell lines with either truncated or mutant p53. In the three cell lines with functional p53 (MCF-7, ZR75-1 and HCC1937), p21 was transactivated following exposure to DACH-acetato-Pt, whereas no significant increase in levels of p21 was observed after 24h exposure to cisplatin. Instead, a decrease in levels of p21 after cisplatin treatment was observed. After 48 hours exposure to cisplatin, p21 was also transactivated in the three cell lines (data not shown), which is indicative of a delayed effect. On the other hand, p21 decreased after exposure to both cisplatin and DACH-acetato-Pt in cell lines with upregulation of basal p21 in functionally-negative p53 group. These observations did not change when the exposure time was extended to 48h (data not shown).

Time course change in G1 cell cycle regulators in ZR75-1 and T47D exposed to the platinum agents. In order to assess in detail the change in the levels of p21 and cell cycle-related proteins, the cisplatin-resistant wild-type p53 ZR75-1 and mutant p53 T47D cell lines were treated with 10μM of each drug for various lengthes of time and protein extacts were subjected to Western immunoblot analysis (Figure5). Time-dependent induction of p53 was observed in ZR75-1 cells exposed to each drug. However, p53 was induced more rapidly in cells exposed to DACH-acetato-Pt than in cells exposed to cisplatin. The rapid transactivation of p21 was evident with DACH-acetato-Pt, whereas, in contrast, a time-dependent decrease in levels of p21 was seen with cisplatin. Decrease in p21 was also observed with time in the T47D cell line exposed to

each drug. Interestingly, expression of Cyclin D1 was decreased in parallel with p21 in both cell lines, although no significant change was observed in levels of Cyclin D1 in ZR75-1 treated with DACH-acetato-Pt. There were no gross changes in Cyclin E and p27 in both cell lines treated with each drug. Thus, the expression of the five genes was not consistent with the greater cytotoxicity of DACH-acetato-Pt relative to cisplatin in both cell lines.

Dose-response change in the levels of p21 and Cyclin D1 and G1/S progression of cell cycle. To investigate if the changes in p21 and Cyclin D1 levels affect the cell cycle, ZR75-1 and T47D cells were treated with a range of concentrations of cisplatin or DACH-acetato-Pt, and the expressed levels of the proteins and cell cycle phase distributions were analyzed. Decrease in the levels of p21 was evident in ZR75-1 cells exposed to lower concentrations of cisplatin, whereas the levels of p21 was increased at lower concentrations of DACH-acetato-Pt, but decreased at higher concentrations (Figure 6A). The levels of Cyclin D1, on the other hand, were decreased in ZR75-1 cells exposed to cisplatin in a concentration-dependent manner, while higher concentrations of DACH-acetato-Pt were needed for the decrease in levels of Cyclin D1. In mutant p53 T47D cell line, the nadir of p21 expression was seen with 10 μM cisplatin and 20 μM DACH-acetato-Pt (Figure 6B). The difference may be due to the relative levels of platinum-DNA adducts formed. Similarly, a decrease in the levels of Cyclin D1 was severe even at the lowest concentration of cisplatin, while the effect of DACH-acetato-Pt was relatively mild. Cell cycle analysis revealed that the changes in p21 expression correlated with the changes in S-phase fraction (SPF) of the cell cycle. The correlation was more significant in T47D cell line with mutant p53 than in ZR75-1 cell

line with wild-type p53. In T47D cells, the decrease in p21 expression showed an inverse pattern to the increase in SPF (Figure 7A-C). In the ZR75-1 cell line, the inverse relationship between p21 levels and changes in SPF is also observed, except high concentration ranges of DACH-acetato-Pt at 48h time point (Figure 7D-F). High concentrations of DACH-acetato-Pt arrested ZR75-1 cells in G1-phase, although the significant decrease in p21 was observed with 24h exposure.

Effects of ubiquitin-proteasome inhibitor on the decrease in p21 and Cyclin D1. In order to examine the possible role of ubiquitin-proteasome degradation pathway for the decrease in p21 and Cyclin D1 proteins, ZR75-1 and T47D cell lines were pretreated with 20 μM N-Acetyl-Leu-Leu-Norleu-al (LLnL; a 26S proteasome inhibitor) following exposure to low (10 μM) or high (50μM) concentration of cisplatin or DACH-acetato-Pt for 16 hours. A low concentration of cisplatin and a high concentration of DACH-acetato-Pt without pretreatment of LLnL induced significant decrease of p21. However, the levels of p21 expression in LLnL-pretreated cells were similar between control and platinum-treated cells, suggesting that the alterations in p21 was related to the ubiquitin-proteosome pathway. On the other hand, the decrease in Cyclin D1 was not affected by LLnL. These results indicate that different mechanisms are involved in the decrease in Cyclin D1 and p21.

DISCUSSION

p21 is a major negative regulator of the G1 checkpoint by binding to and inhibiting the activities of most cyclin/CDK complexes {Sherr & Roberts 1999 54 /id}. Basal level of p21 is reported to be upregulated in some of breast cancer cell lines and tumor samples, in related to some genetic backgrounds. One of such factors is the status of estrogen receptor (ER). Reed et al.{Reed, Florems, et al. 1999 55 /id} investigated 77 node-negative breast cancer samples with immunohistochemistry and revealed that p21 overexpression was associated with positive ER status. Chen et al.{Chen, Lowe, et al. 1999 49 /id} examined 9 breast cancer cell lines and 60 breast tumor samples by Western blot analysis to detect p21, p27 and ER, and found that a strong association between the levels of p21 and ER in both cell lines and tumor samples. On the other hand, both HER2/neu signaling {Yu, Jing, et al. 1998 26 /id} and EGFR signaling {Bromberg, Fan, et al. 1998 57 /id}are reported to increase p21 protein levels. In our panel of breast cancer cell line, there were four cell lines with upregulation of basal p21, two of which were ER-positive with moderate overexpression of HER2/neu (ZR75-1 and T47D){Spink, Spink, et al. 1998 56 /id}, the MDA-MB-330 cell line demonstrated signifiacnt overexpression of both HER2/neu (this study) and EGFR (information from ATCC) and the MDA-MB-157 cell line had none of them. On the other hand, the ER-positive MCF-7 cell line and the SK-Br3 cell line with significant overexpression of HER2/neu demonstrated no evident overexpression of p21, suggesting that cause of the p21 upregulation in breast cancer cells was multifactorial.

In this study, we have investigated the sensitivity to cisplatin and DACH-acetato-Pt in the panel of breast cancer cell lines, and revealed that the basal levels of p21

correlated with the resistance to cisplatin. The effect of increased basal p21 level in tumor cells on the sensitivity to cisplatin is still controversial. For instance, Lincet et al.{Lincet, Poulain, et al. 2000 32 /id} reported that p21 gene transfer into SK-OV-3 and OVCAR3 ovarian cancer cell lines enhanced susceptibility to cisplatin-induced apoptosis. In contrast, several groups have reported the opposite results. Fan et al. {Fan, Chang, et al. 1997 15 /id} has reported that HCT-116 colon cancer cell line and murine embrionic fibroblast with disrupted p21 genes demonstrated significant sensitivity to cisplatin. They have also presented the reduced DNA repair activites in p21-disrupted cell lines. Ruan et al. {Ruan, Okcu, et al. 1999 18 /id} introduced antisense p21 adenovirus expression vector into glioma cell lines, and demonstrated that attenuation of p21 increased sensitivity to BCNU and cisplatin.

On the other hand, the cytotoxicity of DACH-acetato-Pt in breast cancer cell lines was dependent on the presence of functional p53, as we have previously reported with the ovarian cancer panel {Hagopian, Mills, et al. 1999 45 /id}. This compound was very efficient in inducing p53 and p21 in cisplatin-resistant ovarian tumor models {Hagopian, Mills, et al. 1999 45 /id}. In this study, DACH-acetato-Pt has also demonstrated its activity in inducing p21 in cell lines with wild-type p53 function. In contrast, it was surprising results that p21 was significantly decreased in all four cell lines with basal upregulation of p21 following exposure to cisplatin. Similar response in p21 was also observed with DACH-acetato-Pt in three cell lines lacking functional p53 with upregulation of basal p21. These results are the first evidence that p21 is down-regulated with exposure to platinum agents. Wang et al. {Wang, Fan, et al. 1999 48 /id} have reported that ultrviolet (UV) radiation down-regulates p21 expression in a variety of

human cancer cell lines independently of p53 status, while ionizing radiation (IR) caused up-regulation of p21 in cells with wild-type p53 and little or no change in p21 in cells with mutant p53. On the other hand, Park et al. {Park, Carter, et al. 1999 1 /id} reported that IR also initiate negative signals toward the p21 promotor. These results suggest that cisplatin and UV activate similar signaling pathways that are distinct from those activated by DACH-acetato-Pt and IR, as we have mentioned previously {Hagopian, Mills, et al. 1999 45 /id} {Siddik, Mims, et al. 1998 52 /id}.

Western immunoblot analysis of the time-course treatment of the ZR75-1 and T47D cell lines with each drug have revealed that the expression levels of cyclin D1 were also decreased in parallel with p21. As both p21 and cyclin D1 are major cell cycle regulators, especially in G1/S checkpoint, the down-regulation of these protein molecules in response to platinum agents might induce cell cycle progression. Therefore, we have investigated dose-dependent analysis on the changes in p21 and cell cycle distribution. As a result, we found inverted correlation between the relative expression of p21 and the changes in SPF, suggesting that the decrease in p21 may contribute G1/S transition. Recently, in addition, some kinds of DNA damage are reported to increase activity of checkpoint kinases Chk1 and Chk2, which inhibit the kinase activity of cdk2 through phosphorylation, resulting in S-phase arrests {Sampath & Plunkett 2001 58 /id}. In fact, cisplatin-induced DNA damage is reported to activate Chk2 {Damia, Filiberti, et al. 2001 59 /id} and induce S-phase arrest {Hagopian, Mills, et al. 1999 45 /id}. These findings suggest that not only increased G1/S transition but also inhibition of S-phase progression contribute to the accumulation of the cells into S-phase. A checkpoint response induced by cytotoxic agents can have both positive and negative effects on the induction of

apoptosis {Sampath & Plunkett 2001 58 /id} {Arooz, Yam, et al. 2000 2 /id}, and the relationship between the S-phase accumulation and the induction of apoptosis is still unclear. However, the increase in SPF in response to platinum agents seems to affect the resistance in these cell lines.

Interestingly, concentration effects were observed in the decrease in p21 in response to either drug. Lower concentrations of cisplatin demonstrated strong effect on the decrease in p21 protein, whereas high concentrations of DACH-acetato-Pt decreased p21 significantly. The difference in the effects on p21 between the drugs may be due to the difference in the extent of DNA adducts. On the other hand, in wild-type p53 ZR75-1 cells, lower concentrations of DACH-acetato-Pt induced p21 more efficiently than the same concentrations of cisplatin through the rapid induction of p53. As a result, there observed a big difference between cisplatin and DACH-acetato-Pt in the levels of p21 of the ZR75-1 cells treated with lower concentrations of each drug. The difference in the expression of p21 may explain more than 10-fold difference in DNA damage tolerance to cisplatin and DACH-acetato-Pt in ZR75-1 cell line.

We have pretreated cells with a proteasome inhibitor LLnL prior to drug exposure and revealed that the decrease in p21 was, in some extent, due to proteasomal degradation. However, LLnL failed to inhibit the decrease in cyclin D1, suggesting that there are different mechanisms involved in the decreased in p21 and cyclin D1. In addition, decrease in the levels of cyclinD1 was never observed in ZR75-1 cells treated with lower concentrations of DACH-acetato-Pt, where the p21 levels were higher than the basal level, as shown in Figure 5A and 6A. These results suggest that the decrease in cyclin D1 may be a secondary event following the down-regulation of p21.

In conclusion, the upregulation in basal levels of p21 in breast cancer cell lines correlated with cisplatin-resistance, while DACH-acetato-Pt was still effective against such tumor cells. On the other hand, the cytotoxicity of DACH-acetato-Pt was dependent on the presence of functional p53. These results suggest that DACH-acetato-Pt may have clinical potential against breast tumors, especially with wild-type p53 background.

MS #2

Table 1. p53 status of breast cancer cell lines

Cell line	p53 gene status	DNA sequence	amino acid	References
MCF-7	wild-type			1
ZR75-1	wild-type			This study
HCC1937	mutant	306: CGA to TGA	Arg to Term	2
MDA-MB-330	mutant	220: TAT to TGT	Tyr to Cys	This study
MDA-MB-435	mutant	266: GGA to GAA	Gly to Glu	3
SK-Br3	mutant	175: CGC to CAC	Arg to His	4
T47D	mutant	194: CTT to TTT	Leu to Phe	~~5~~ 3
MDA-MB-157	null			~~6~~ 5
MDA-MB-436	null			~~6~~ 5

Fan, S Cancer Res 55:1649

Tomlinson, GE Cancer Res 58:323

O'connor PM Cancer Res 57 4285

Sheikh, MS Int J Radiat Biol.

Chen X Oncogene 18.5691

Table 2. IC_{50} of cisplatin and DACH-acetato-Pt in breast cancer cell lines following continuous drug exposure

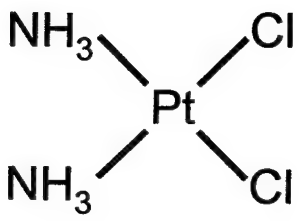
Cell line	IC_{50} value (μM)		Cisplatin/DACH-acetato-Pt ratio
	Cisplatin	DACH-acetato-Pt	
functional p53 (+)			
MCF-7	0.83 ± 0.27*	0.17 ± 0.12	4.88
ZR75-1	9.58 ± 1.59	0.85 ± 0.29	11.27
HCC1937	0.65 ± 0.11	0.23 ± 0.05	2.83
functional p53 (-)			
MDA-MB-330	7.43 ± 2.55	1.10 ± 0.44	6.75
MDA-MB-435	0.84 ± 0.06	1.11 ± 0.36	0.76
SK-Br3	0.59 ± 0.24	1.38 ± 0.09	0.43
T47D	4.89 ± 0.30	1.84 ± 0.52	2.66
MDA-MB-157	0.75 ± 0.23	1.29 ± 0.49	0.58
MDA-MB-436	0.16 ± 0.12	0.25 ± 0.14	0.64

* Mean ± SD; n=3-5

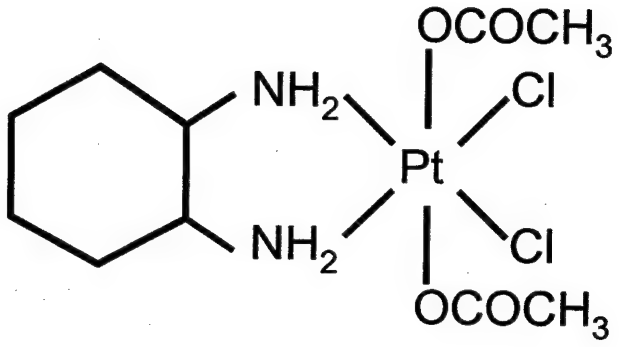
Table 3. Biochemical pharmacology of cisplatin and DACH-acetato-Pt against ZR75-1 and T47D breast cancer cell lines

Cell Line	ZR75-1		T47D	
Drugs	cisplatin	DACH-acetato-Pt	cisplatin	DACH-acetato-Pt
2-h platinum uptake (ng Pt/mg protein)	71.4 ± 5.3*	58.0 ± 13.5	59.8 ± 9.4	63.3 ± 8.6
DNA adducts at 2-h (ng Pt/mg DNA)	30.6 ± 7.1	20.4 ± 2.6	22.7 ± 3.0	9.3 ± 0.7
DNA damage tolerance (ng Pt/mg DNA at IC_{50})	2.9 ± 0.7	0.17 ± 0.02	1.1 ± 0.2	0.17 ± 0.01

* Mean ± SD



Cisplatin



1R,2R-DACH-$(Ac)_2Cl_2$-Pt(IV)

Figure 1

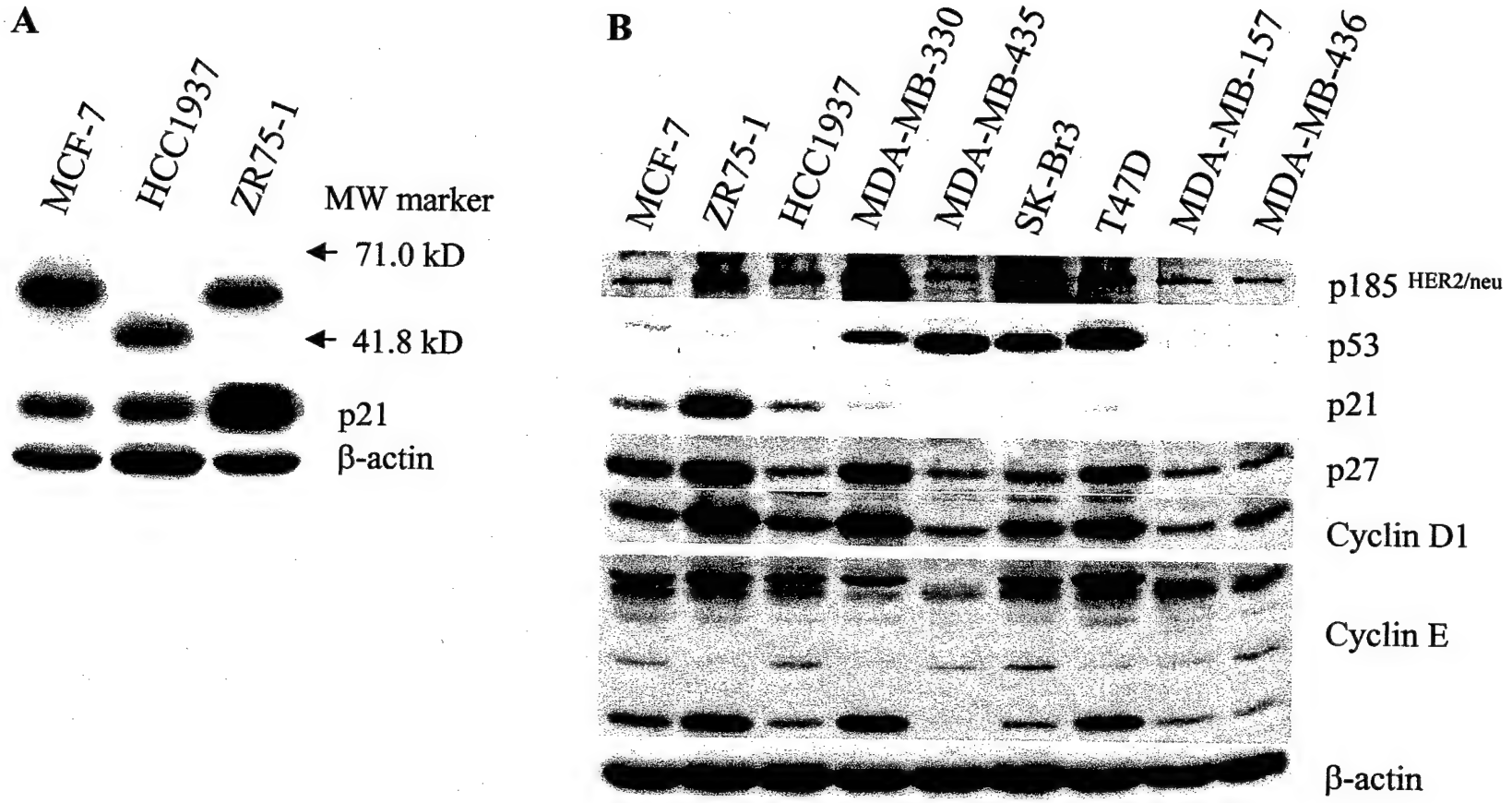

A
MCF-7
HCC1937
ZR75-1
MW marker
71.0 kD
41.8 kD
p21
β-actin
B
MCF-7
ZR75-1
HCC1937
MDA-MB-330
MDA-MB-435
SK-Br3
T47D
MDA-MB-157
MDA-MB-436
p185 HER2/neu
p53
p21
p27
Cyclin D1
Cyclin E
β-actin

Figure 2

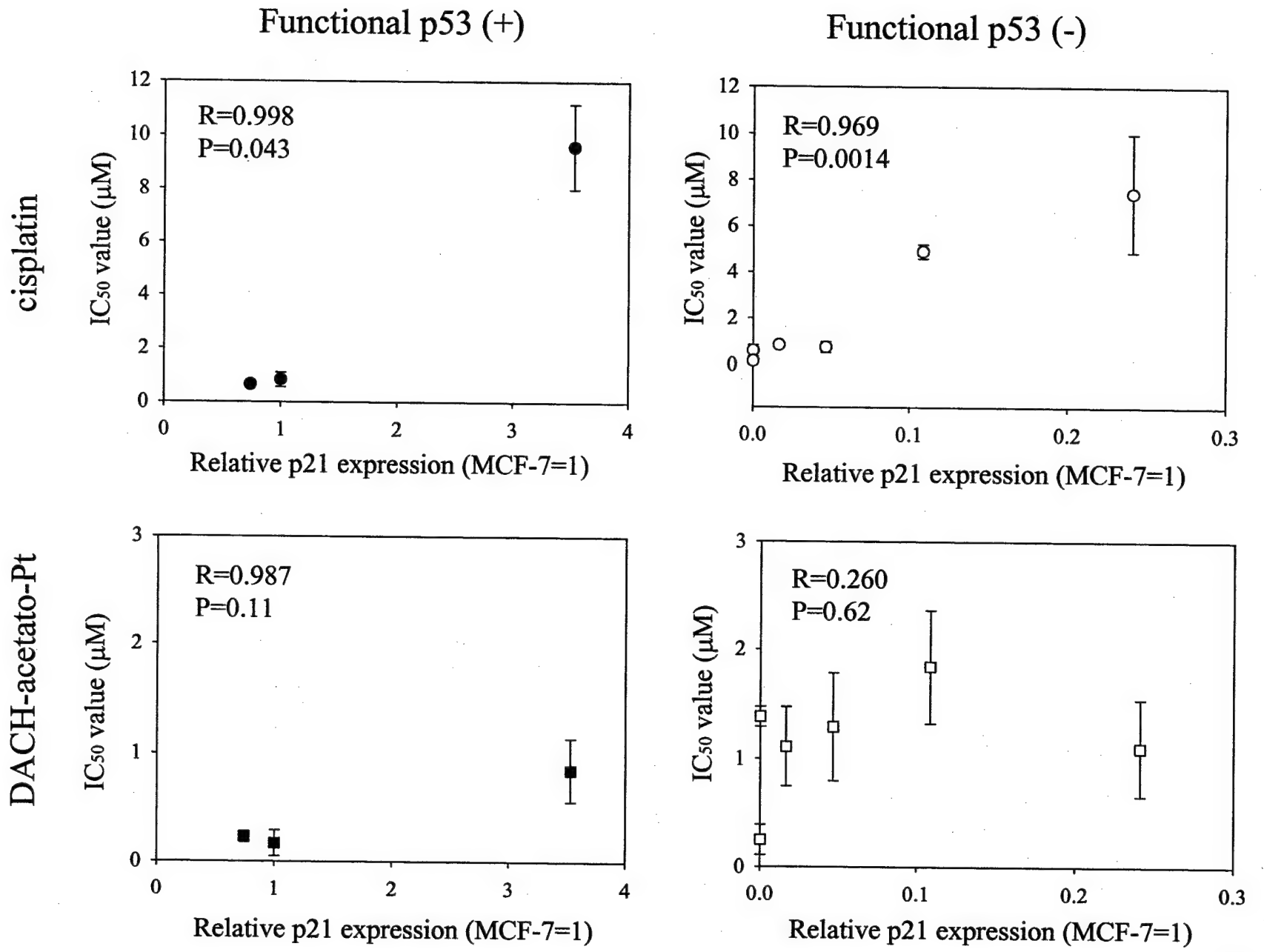

Functional p53 (+)
Functional p53 (-)
cisplatin
DACH-acetato-Pt
IC50 value (μM)
Relative p21 expression (MCF-7=1)
R=0.998
P=0.043
R=0.969
P=0.0014
R=0.987
P=0.11
R=0.260
P=0.62

Figure 3

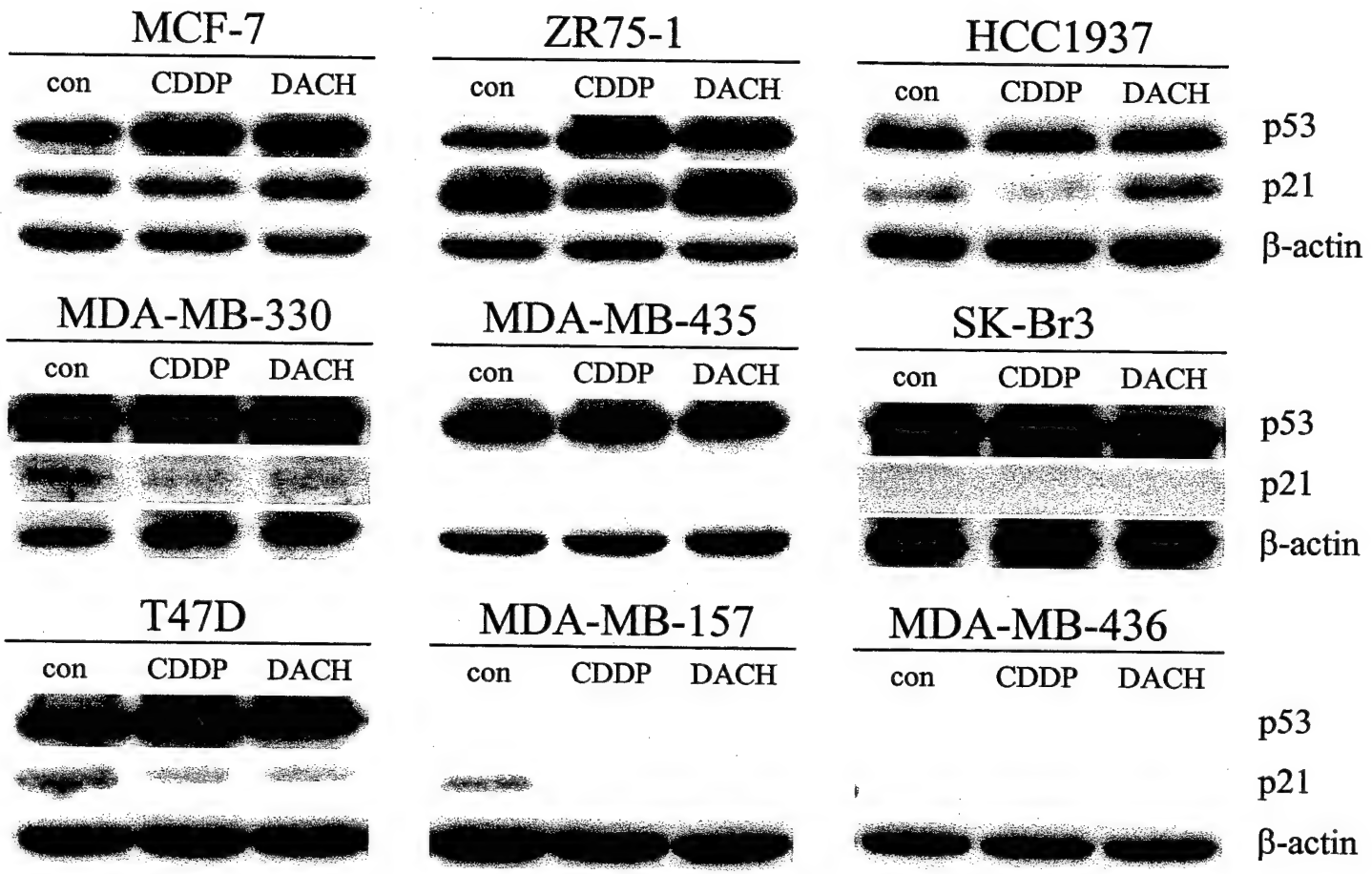

MCF-7
con
CDDP
DACH
ZR75-1
con
CDDP
DACH
HCC1937
con
CDDP
DACH
p53
p21
β-actin
MDA-MB-330
con
CDDP
DACH
MDA-MB-435
con
CDDP
DACH
SK-Br3
con
CDDP
DACH
p53
p21
β-actin
T47D
con
CDDP
DACH
MDA-MB-157
con
CDDP
DACH
MDA-MB-436
con
CDDP
DACH
p53
p21
β-actin

Figure 4

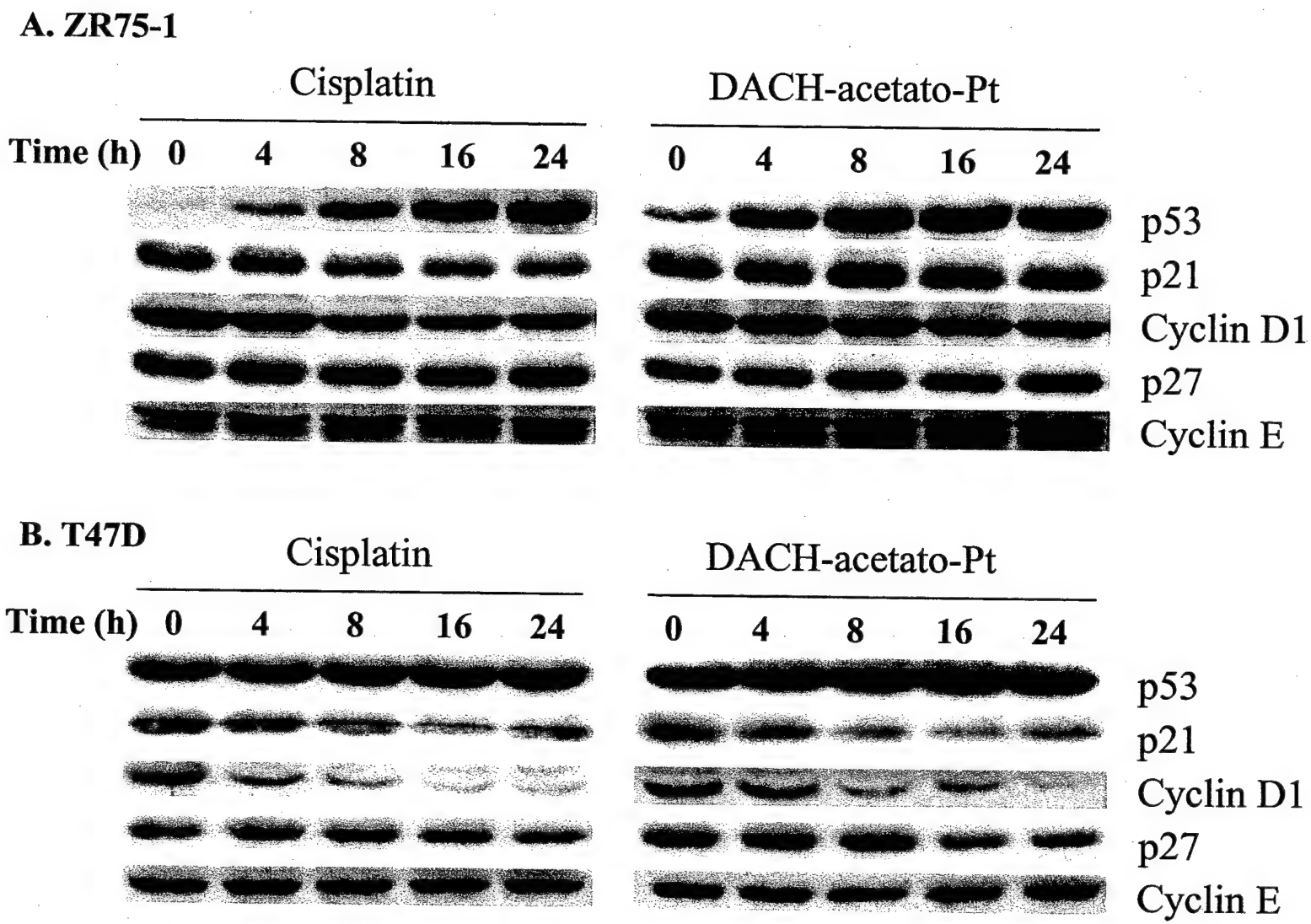

A. ZR75-1
Cisplatin
DACH-acetato-Pt
Time (h) 0 4 8 16 24
0 4 8 16 24
p53
p21
Cyclin D1
p27
Cyclin E
B. T47D
Cisplatin
DACH-acetato-Pt
Time (h) 0 4 8 16 24
0 4 8 16 24
p53
p21
Cyclin D1
p27
Cyclin E

Figure 5

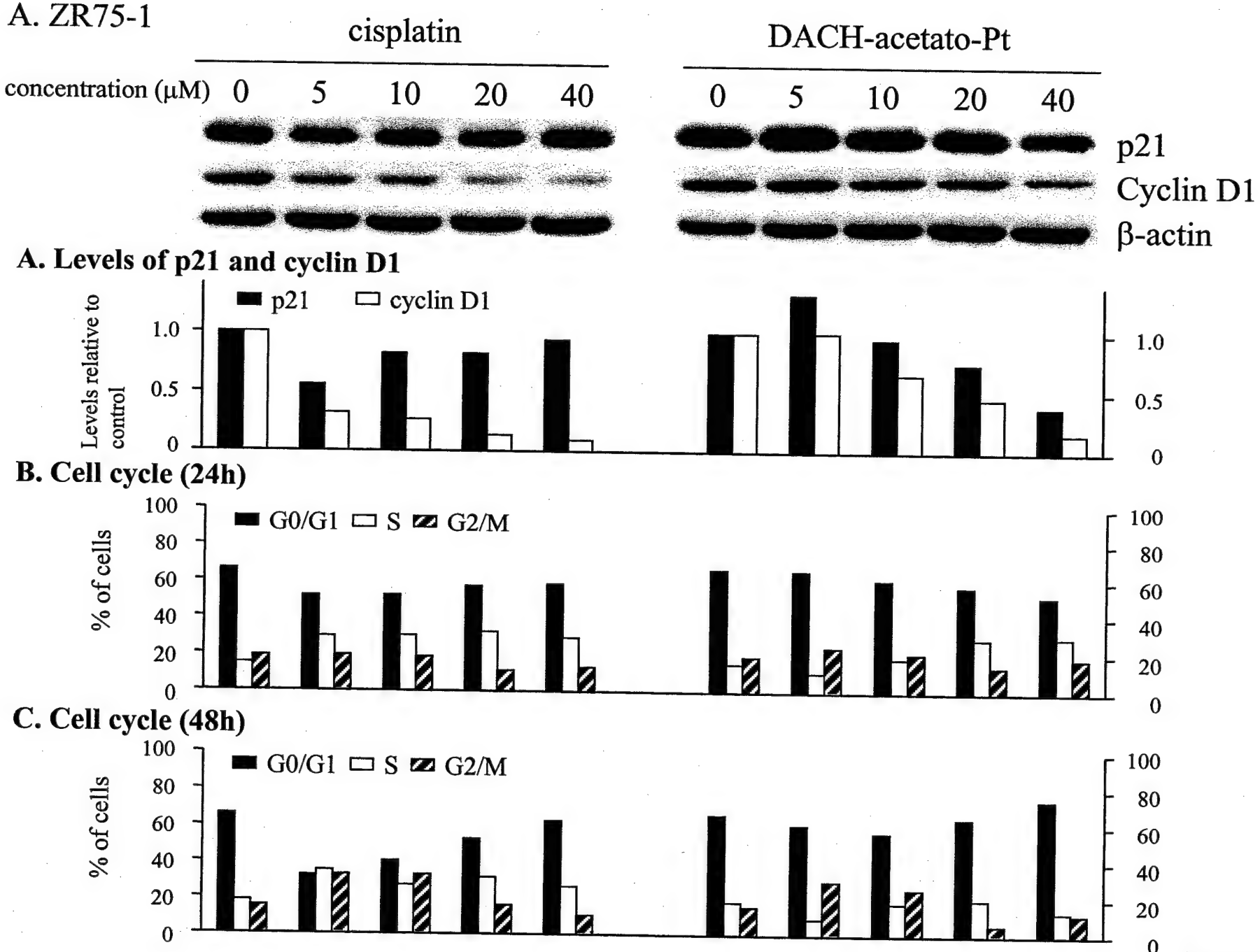

A. ZR75-1
cisplatin
DACH-acetato-Pt
concentration (μM) 0 5 10 20 40
0 5 10 20 40
p21
Cyclin D1
β-actin
A. Levels of p21 and cyclin D1
p21
cyclin D1
Levels relative to control
1.0
0.5
0
B. Cell cycle (24h)
G0/G1
S
G2/M
% of cells
100
80
60
40
20
0
C. Cell cycle (48h)

Figure 6A

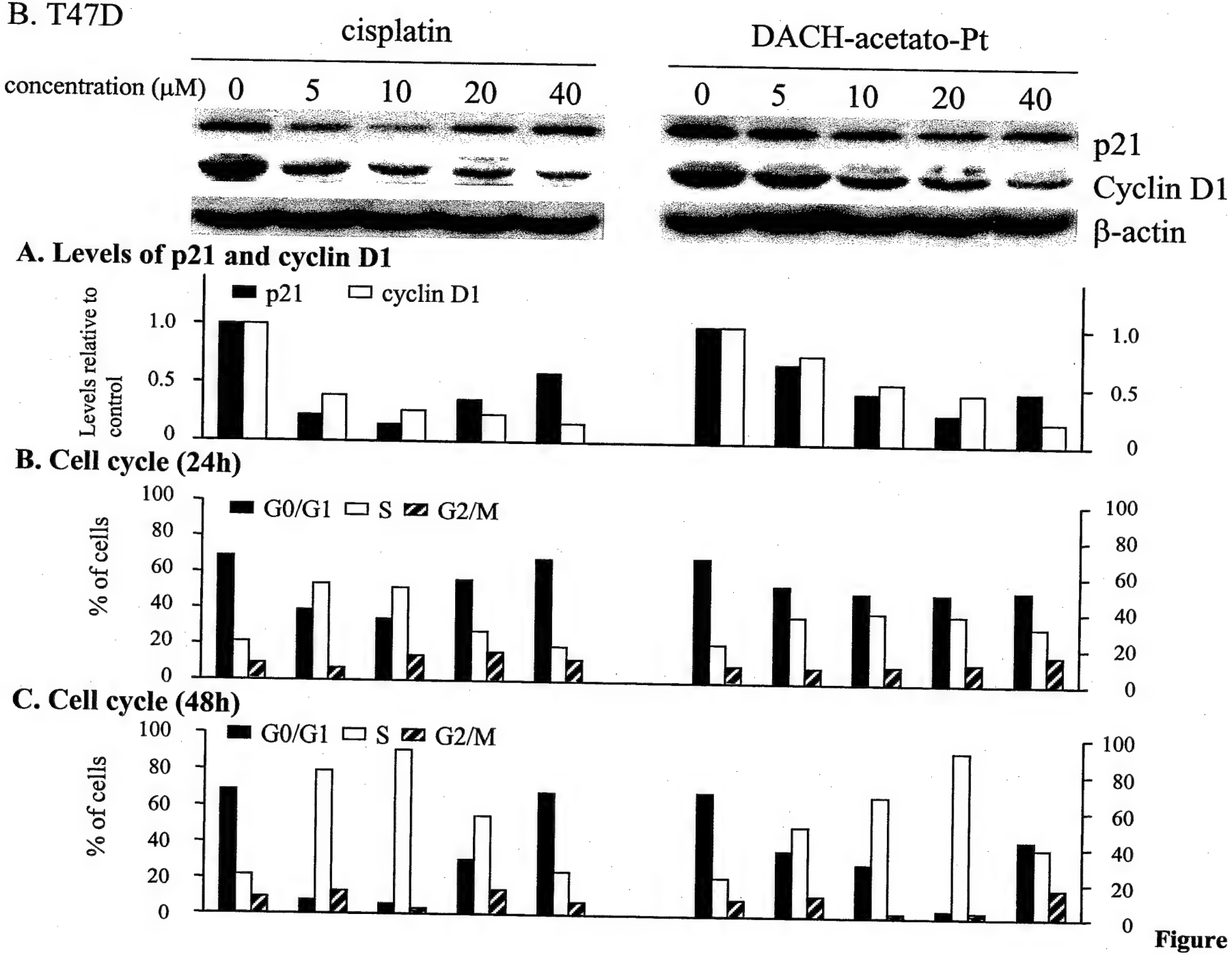

B. T47D
cisplatin
DACH-acetato-Pt
concentration (μM) 0 5 10 20 40
0 5 10 20 40
p21
Cyclin D1
β-actin
A. Levels of p21 and cyclin D1
Levels relative to control
p21
cyclin D1
1.0
0.5
0
B. Cell cycle (24h)
% of cells
G0/G1
S
G2/M
100
80
60
40
20
0
C. Cell cycle (48h)
% of cells
G0/G1
S
G2/M

Figure 6B

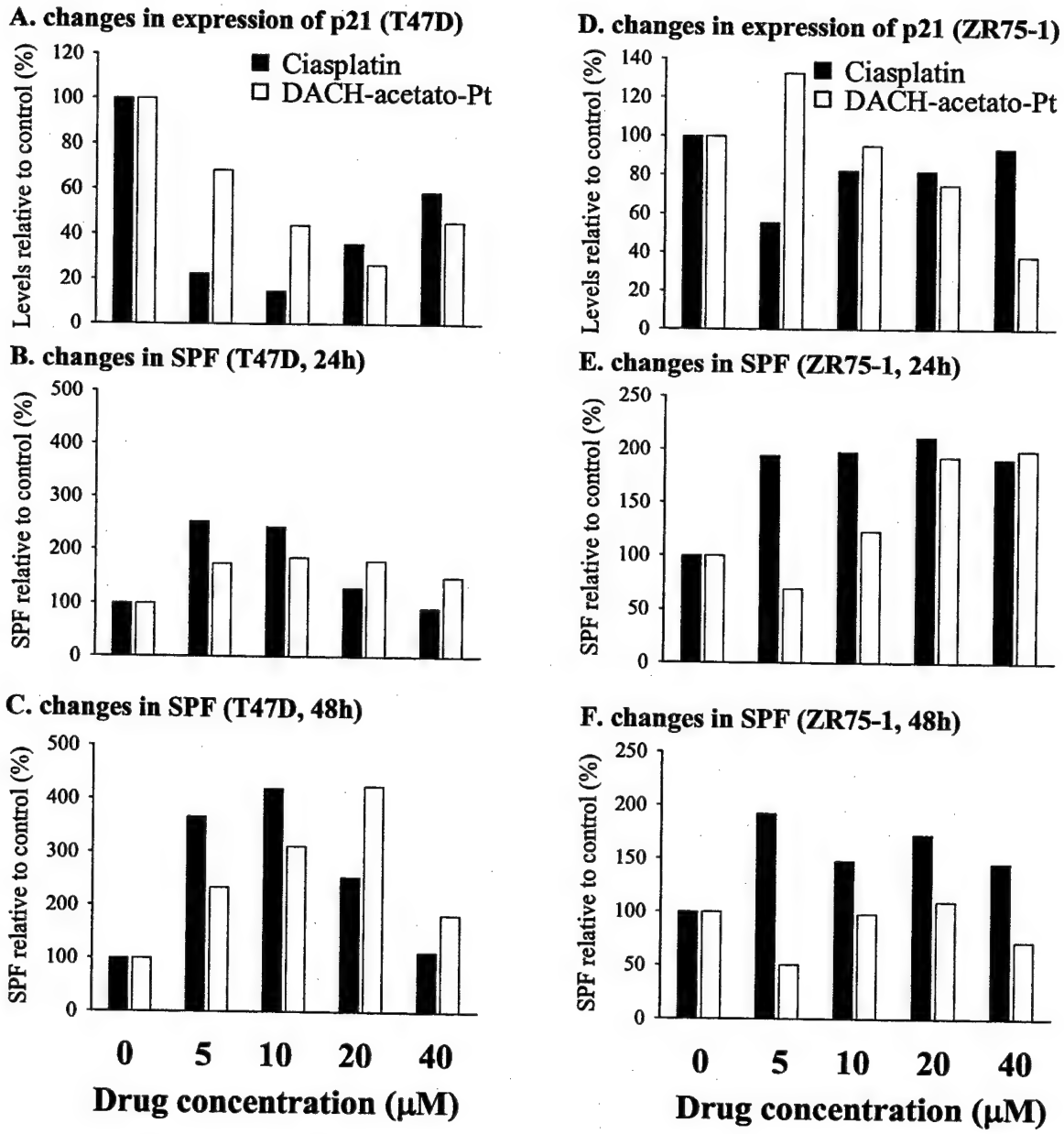

A. changes in expression of p21 (T47D)
Levels relative to control (%)
120
100
80
60
40
20
0
Ciasplatin
DACH-acetato-Pt
B. changes in SPF (T47D, 24h)
SPF relative to control (%)
500
400
300
200
100
0
C. changes in SPF (T47D, 48h)
SPF relative to control (%)
500
400
300
200
100
0
0 5 10 20 40
Drug concentration (μM)
D. changes in expression of p21 (ZR75-1)
Levels relative to control (%)
140
120
100
80
60
40
20
0
Ciasplatin
DACH-acetato-Pt
E. changes in SPF (ZR75-1, 24h)
SPF relative to control (%)
250
200
150
100
50
0
F. changes in SPF (ZR75-1, 48h)
SPF relative to control (%)
250
200
150
100
50
0
0 5 10 20 40
Drug concentration (μM)

Figure 7

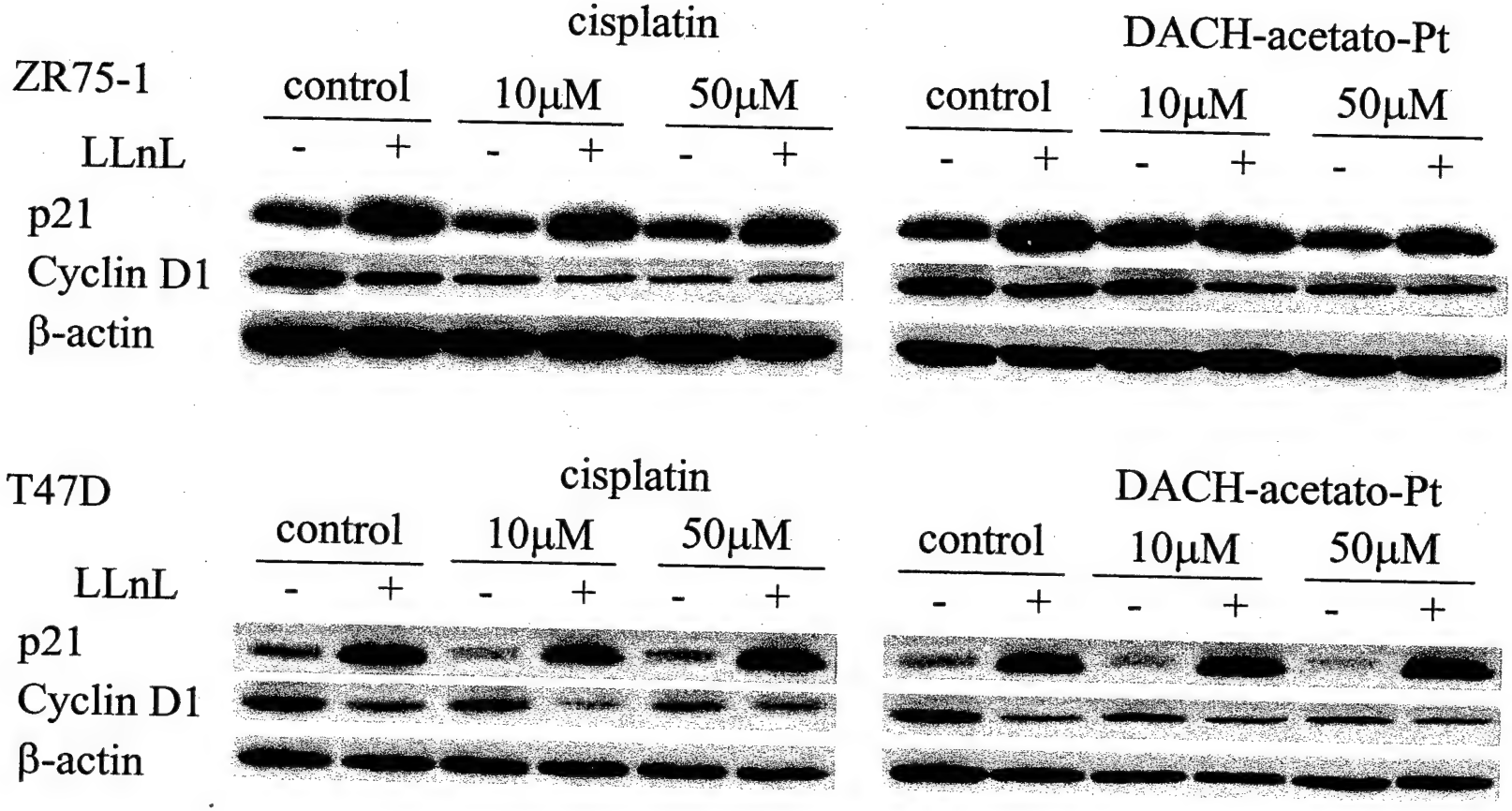

cisplatin
DACH-acetato-Pt
ZR75-1
control
10μM
50μM
LLnL
- + - + - +
p21
Cyclin D1
β-actin
T47D
cisplatin
DACH-acetato-Pt
control
10μM
50μM
LLnL
- + - + - +
p21
Cyclin D1
β-actin

Figure 8

APPENDIX – MS #3 (in preparation)

Wild-type p53 reduces DNA-damage tolerance of HER2/neu-overexpressing ovarian cancer cells in a drug-dependent manner

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Running Title: p53 and platinum against HER2-overexpressing tumor

Key words: HER2/neu, p53, platinum, ovarian cancer, DNA damage tolerance.

FOOTNOTES

[1] This work was supported by U.S. Army Grant DAMD 17-99-1-9269, and NCI RO1 CA77332 and RO1 CA82361.

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[3] The abbreviations used are: EGFR, epidermal growth factor receptor; DACH-acetato-Pt, 1R,2R-diaminocyclohexan-diacetato-dichloro-platinum (IV); FBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide.

ABSTRACT

In order to evaluate the effects of p53 on the cytotoxicity of cisplatin and novel analog DACH-acetato-Pt against HER2/neu-overexpressing tumor cells, we established clones by transfecting the null-p53, HER2/neu-overexpressing SK-OV-3 ovarian tumor model with a temperature-sensitive mutant (TS) p53. At 37 °C when TS-p53 functioned as a mutant, drug sensitivity of the TS9 clone with low p53 expression was unaffected, whereas the TS4 clone with high p53 expression demonstrated up to 2-fold resistance to cisplatin (IC_{50}: 0.84 vs. 1.63 μM), suggesting that the mutant p53 has gain-of-function on cell survival. At 32 °C when TS-p53 functioned as a wild-type, sensitivity to cisplatin was comparable between TS and neo, but the sensitivity to DACH-acetato-Pt was increased 2- to 5-fold in TS clones (IC_{50}: 14.3 vs. 5.91 μM in clone 4, IC_{50}: 22.7 vs. 4.63 in clone 9). Modulation of cytotoxicity of TS4 cells was not due to changes in intracellular drug uptake or the formation of DNA adducts. However, the data indicated that functional p53 either normalized or decreased tolerance to DNA damage induced by the platinum agents. Western analysis demonstrated that cisplatin-induced DNA damage significantly activated the Akt pathway through increased phosphorylation of $p185^{HER2/neu}$, whereas the effect of DACH-acetato-Pt on $p185^{HER2/neu}$ phosphorylation was transient and limited. Although at 32 °C p53 was induced to a similar extent by each drug, p21 was transactivated more efficiently by DACH-acetato-Pt than by an equitoxic concentration of cisplatin in TS4. These results suggest that the inhibition of wild-type p53 function is associated with increased levels of phosphorylated $p185^{HER2/neu}$. Our studies provide evidence that activation of functional p53 not only normalizes sensitivity of cells overexpressing HER2/neu to cisplatin by circumventing the dominant-negative

effects of mutant p53, but also increases sensitivity to DACH-acetato-Pt by over-riding the effects of HER2/neu overexpression. Thus, a combination of wild-type p53 and DACH-acetato-Pt may be an effective treatment against tumors overexpressing HER2/neu.

INTRODUCTION

HER2/neu encodes a 185 kD transmembrane glycoprotein which is a member of the type I receptor tyrosine kinase family with homology to EGFR[3] (1). This gene is often amplified and/or overexpressed in a variety of human tumors (2,3), indicating its critical role in the development of human cancers. In breast and ovarian cancers, for instance, the incidence of HER2/neu amplification is reported to be 10-34 % (4) and 19-59 % (5), respectively. Amplification/overexpression of this oncogene is also reported to correlate with poor prognosis, as it enhances the metastatic potential of cancer cells (6) and induces resistance to various chemotherapeutic agents (7,8).

The antitumor agent cisplatin is highly effective in the clinic against human cancers (9,10). However, the presence of primary or the emergence of secondary resistance significantly undermines the clinical utility of this drug (11). Several reports indicate that amplification/overexpression of HER2/neu can directly reduce cisplatin cytotoxicity (12,13). Considering that tumor cells rarely have a single genetic defect, it needs to be recognized that the presence of other genetic abnormalities may also contribute to the overall poor prognosis. One such abnormality relates to the p53 gene. In fact, HER2/neu amplification was found in 41% of the samples with p53 abnormalities as compared to 15.9% of the samples without p53 abnormalities (14). The tumor suppressor p53 is mutated or deleted in over 50% of all human tumors (15). As 'guardian of genome', wild-type p53 mediates cell-cycle arrest or apoptosis in response to DNA damage (16), and loss of p53 function is associated with resistance to apoptosis induced by chemotherapy and radiotherapy (17,18). Recently, several preclinical studies suggest that transfer of wild-type p53 combined with conventional doses of radiation or

chemotherapeutic agents may have a synergistic effect without additional toxicity (19,20).

Recent studies suggest that there may be a link between signal transduction pathways for HER2/neu and p53 (21,22). Casalini et al. (22), for instance, have demonstrated that HER2/neu overexpression led to tumor cell proliferation in the IGROV1 /Pt1 model with mutant p53, but resulted in apoptosis in IGROV1 cells harboring wild-type p53. Similarly, the ras-mediated signal transduction pathway, which is a downstream target of HER2/neu signal (23), inhibits p53 function (24) and is associated with resistance to therapeutic DNA damaging agents (25). On the other hand, Zhou et al. (26) reported that overexpression of HER2/neu induced MDM2 phosphorylation through the phosphatidylinositol-3'-OH kinase (PI3K)- Akt pathways and the phosphorylated MDM2 increased p53 ubiquitination. We have demonstrated that the DNA damaging antitumor agent cisplatin and the non-cross-resistant analog 1R,2R-diaminocyclohexane-diacetato-dichloro-platinum (IV) (DACH-acetato-Pt; Figure 1), were less cytotoxic against the ovarian tumor SK-OV-3 model (27), which overexpresses HER2/neu and is null for p53 (28). However, the analog was very effective against cisplatin-resistant tumor cell lines harboring wild-type p53 (27). Therefore, it is possible that the lack of effectiveness of DACH-acetato-Pt against SK-OV-3 cells may be due to the absence of p53.

Based on the above information, we hypothesized that introduction of functional p53 in combination with DACH-acetato-Pt could be an effective treatment against tumors overexpressing HER2/neu. In order to test the hypothesis, we transfected SK-OV-3 with a temperature-sensitive (TS) p53 vector which assumes mutant p53 configuration at 37

°C and wild-type at 32 °C (29). We report here that the cytotoxicity of DACH-acetato-Pt was significantly enhanced in the presence of functional p53, although it did not alter the impediment of HER2/neu overexpression on cisplatin-mediated cytotoxicity.

MATERIALS AND METHODS

Chemicals. Cispaltin was obtained from Sigma Chemical Co. (St.Louis, MO). We have previously reported the synthesis and chemical characterization of DACH-acetato-Pt (30). Cisplatin and DACH-acetato-Pt were dissolved in normal saline and water, respectively, then sterilized through 0.22-μm disc filter. The concentration of each drug was confirmed by flameless atomic absorption spectroscopy (FAAS) (31). MTT was purchased from Sigma Chemical Co. (St. Louis, MO).

Cell Culture and Transfection. SK-OV-3, which was originally established from malignant ascites of a patient treated with thiotepa (32), was obtained from American Type Culture Collection (Rockville, MD), and maintained in McCoy's 5A supplemented with 10% heat-inactivated FBS, 2 mM L-glutamin, and antibiotics under a humidified atmosphere of 5% CO_2. SK-OV-3 lacks expression of p53 mRNA and protein, and overexpresses HER2/neu (28). pC53NN M133T, which is a plasmid containing a TS-p53 cDNA under the control of a CMV promotor was kindly provided by Dr. Guillermina Lozano (The University of Texas, M.D. Anderson Cancer Center, Houston, TX). The plasmid was introduced into cells using FuGENE 6 transfection reagent. After 14 days culture in the selective medium containing 600 μg/ml G418, G418-resistant colonies were selected and expanded. The clones were examined for their stable growth characteristics and expression of p53. Two clones with different levels of p53 expression (TS4 and TS9) were used in this study. Two G418-resistant sub-clones derived from TS4 and TS9 that no longer expressed p53 were used as controls (neo4 and neo9, respectively). Cells were maintained in McCoy's 5A supplemented with 10% heat-

inactivated FBS, 2 mM L-glutamin, and 600 μg/ml G418, and examined regularly for p53 expression.

Cytotoxicity and Biochemical PharmacologyStudies. Cells were seeded in 96-well plates, allowed to attach for 24 hours and then either maintained for another 24 hours at 37 °C or shifted to 32 °C. Following acclimation, cells were exposed to various concentrations of cisplatin or DACH-acetato-Pt. After 5 days of incubation at 37 °C or 7 days at 32 °C, the sensitivities of the cells to the platinum agents were evaluated using a modified MTT assay (33). Evaluations in attached cells of cellular platinum uptake and DNA adduct formation were conducted as described previously (34,31). Briefly, cells treated with 100μM of cisplatin or DACH-acetato-Pt for 2 hours at each temperature were collected and washed. For determination of cellular uptake, cell pellets were first digested overnight at 55 °C in 50 μl of 1 M hyamine hydroxide (ICN, Irvine, CA). To measure platinum-DNA adduct formation, high molecular weight DNA was isolated from cell pellets according to standard procedures (35). The platinum content of both samples was determined by FAAS. Platinum-DNA damage tolerance, defined as the level of DNA adducts at IC_{50}, was assessed as previously reported (36).

Western Analysis Cells were acclimated at 32 °C for 24 hours prior to drug exposure, and were exposed to 5 x IC_{50} concentrations of cisplatin or DACH-acetato-Pt (29.3 μM cisplatin or 29.6 μM DACH-acetato-Pt) for 48 hours. At 12-hour intervals, cells were collected, washed twice with ice-cold phosphate-buffered saline and lysed for 20 min on ice with 100 μl of lysis buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.02% sodium azide, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate, 100 μg/ml phenylmethylsulfonyl fluoride, and 1 μg/ml aprotinin). The lysates were collected by

microcentrifugation at 4 °C, and then the protein was determined by the standard Lowry procedure. Forty μg of total cell protein was electrophoresed on a 10% SDS-polyacrylamide gel, transferred onto nitrocellulose membranes, and incubated with various antibodies. Mouse monoclonal anti-p53 (Ab-6) was obtained from Oncogene Research Products (Cambridge, MA), and anti-p21 (Cip1/Waf1) antibody was purchased from Transduction Laboratories (Lexington, KY). Rabbit polyclonal anti-phospho-ErbB2 (Y1248) was obtained from upstate biotechnology (Lake Placid, NY). Rabbit polyclonal anti-phospho-p53 antibodies (serine 15 and serine 392), anti-phospho-Akt (serine 473) and anti-phospho-Erk1/Erk2 MAPK (Thr202/Tyr 204) were purchased from Cell Signaling Technology (Beverly, MA). Mouse monoclonal anti-β-actin antibody was purchased from Sigma (St. Louis, MO). All immunoblots were visualized by enhanced chemiluminescence detection system (Amersham, Arlington Heights, IL), and quantified by laser densitometry.

Statistical Analysis. Differences between groups were evaluated by Student's *t*-test.

RESULTS

Establishment of Stable Transfectants. We utilized two TS clones with different levels of p53 expression and the corresponding neo clones, which were used as controls (Figure 2A). All clones showed stable growth, even at 32 °C when p53 functions as wild-type. The expression of p53 in TS4 at 37 °C was almost 3-fold higher than that in TS9, while both neo clones did not express p53. HER2/neu was overexpressed in all clones to the same extent, and the levels of the expression were almost 50-fold higher than that in MCF-7 breast cancer cell line, which was used as a negative control. Basal levels of p53 and expression of p53-dependent transactivation of p21 in TS and neo clones under different temperature conditions are shown in Figure 2B. Expression of p21 was apparent in both of TS clones only at 32 °C, and this was consistent with the understanding that the introduced p53 functioned as wild-type at 32 °C and was able to transactivate the p21 (Waf1/Cip1) gene. The extent of p21 expression was almost 5-fold higher in TS4 at 32 °C than that in TS9.

Cytotoxicity of Cisplatin and DACH-acetato-Pt. The results of cytotoxic evaluation are shown in Table 1. TS4 demonstrated about 2-fold resistance to cisplatin compared to neo4 at 37 °C (IC_{50}: 0.84 vs. 1.63 μM) whereas resistance to DACH-acetato-Pt was not significant (1.58 vs. 2.19 μM). However, there was no significant difference between neo9 and TS9 in the sensitivity to both drugs at 37 °C. These results suggest that high expression levels of mutant p53 increased significant resistance to cisplatin only, while low levels of mutant p53 did not affect the cytotoxicity of the drugs. At 32 °C, on the other hand, there was no significant difference in the cytotoxicity of cisplatin between neo and TS in both clone 4 and clone 9. In contrast, the sensitivity to DACH-

acetato-Pt was significantly increased in both TS4 and TS9 compared to each neo clone (IC_{50}: 14.3 vs. 5.91 μM in clone 4, IC_{50}: 22.7 vs. 4.63 in clone 9), and TS/neo IC_{50} ratio, as an index of resistance, was decreased to 0.41 and 0.20 in clone 4 and 9, respectively. These results indicate that functional p53 increases sensitivity of HER2/neu overexpressing SK-OV-3 cells to DACH-acetato-Pt, although it only normalized or retained the cisplatin cytotoxicity to that seen in control cells.

Biochemical Pharmacology of Cisplatin and DACH-acetato-Pt. In order to rationalize the cytotoxicity data, the biochemical pharmacology of the platinum agents was investigated in neo4 and TS4 at each temperature (Table 2). Cellular uptake of platinum was similar in both cell lines at either temperature. Furthermore, there was no significant difference in DNA adducts between the cell lines. However, at 37 °C, DNA damage tolerance to cisplatin was significantly increased in the TS4 clone compared to neo. In contrast, there was no difference in damage tolerance to cisplatin at 32 °C. On the other hand, DNA damage tolerance to DACH-acetato-Pt was significantly decreased in TS4 compared to neo4 at 32 °C (1.13 vs. 0.51 ng Pt/mg DNA). These results suggest that changes in sensitivity to cisplatin and DACH-acetato-Pt by alterations in functional p53 status correlates with inverse changes in the tolerance to platinum adducts.

Effects of drug-induced DNA damage on HER2/neu signaling and the activation of wild-type p53. In order to understand the differential modulation by wild-type p53 on the cytotoxicity of cisplatin and DACH-acetato-Pt, studies were undertaken to investigate the effect of drug-induced DNA damage on the activation of HER2/neu signaling and activation of p53: TS4 cells were exposed to equitoxic concentrations of

each drug at 32 °C and cell extracts were subjected for Western immunoblot analysis. The activity of HER2/neu signaling was evaluated by phosphorylation status of $p185^{HER2/neu}$ and two major down-stream targets Akt and Erk1/2. On the other hand, the function of p53 is regulated by phosphorylation at several sites on the p53 molecule, including serine 15 and serine 392 (16). Serine 15 phosphrylation appears to be closely associated with induction of p53 through an increase in protein stabilization (16). Therefore, in order to assess the function of introduced p53 protein in response to the platinum agents, the phosphorylation status of p53 and transactivation of p21 were examined. The Western immunoblots and results of densitometric analysis of the immunoblots are shown in Figure 3 and 4, respectively. Although no change in levels of total HER2/neu expression was observed during the time course (data not shown), the active, phosphorylated form of $p185^{HER2/neu}$ was significantly increased and peaked in TS4 cells treated with cisplatin, whereas the increase of phospho-$p185^{HER2/neu}$ in cells exposed to DACH-acetato-Pt was relatively low with a peak effect observed at 12 h (Figure 4A). A significant time-dependent increase in phosphorylation of Akt and decrease in phosphorylation of Erk1/2 was observed in TS4 cells exposed to cisplatin, although a transient, low-level increase in phosphorylation of these proteins was seen with DACH-acetato-Pt (Figure 4B, C). These results inidcate that cisplatin-induced DNA damage activates HER2/neu signaling which induces the PI3K-Akt pathway, whereas DNA damage induced by DACH-acetato-Pt has reduced effect on HER2/neu signaling. The levels of phospho-$p185^{HER2/neu}$ and phospho-Akt were not affected by temperature shift from 37 °C to 32 °C (data not shown). Total p53 was induced with time in TS4 cells exposed to both drugs (Figure 4D). Phosphorylation of p53 at both

serine 15 and serine 392 was evident in cells treated with cisplatin, whereas lesser extent of serine 15 phosphorylation and no detectable levels of phosphorylation at serine 392 were observed in cells exposed to DACH-acetato-Pt. Although the induced levels of total p53 were similar in cells exposed to cisplatin and DACH-acetato-Pt treatment, the levels of p53 phosphorylated at serine 15 were grossly different (Figure 4E). Moreover, the increase in this phosphorylated form of p53 by cisplatin paralleled the increase in total p53, whereas the increase in phosphorylated p53 by DACH-acetato-Pt was observed much later. Changes in levels of total and phospho-p53 and p21 were not observed in either neo4 cells treated with either drug at 32 °C or in TS4 cells treated at 37 °C (data not shown). These results indicate that increase in p53 can also occur through a mechanism not involving serine 15 phosphorylation. Increase in the levels of p21 protein was also seen with time in cells treated with each drug (Figure 4F). These results suggest that the TS p53 acted as wild-type at 32 °C. However, the extent of p21 transactivation was 2-fold higher in cells treated with DACH-acetato-Pt than that with cisplatin. These results suggest that different pathways are involved in p53 activation with the two platinum agents, and that differential post-translational modifications of p53 may explain the different extent of p21 transactivation. It is clear that phosphorylation on neither serine 15 nor serine 392 paralleled the increase in p21 following exposure of cells to DACH-acetato-Pt.

DISCUSSION

Human ovarian SK-OV-3 tumor cells are null for p53 and overexpress HER2/neu (28), and our previous investigations have demonstrated that these molecular impediments cause the SK-OV-3 model to display resistance to cisplatin (11,27). The findings confirmed literature reports (12,13,37,38) and were consistent with the reported demonstration that tumor cells overexpressing HER2/neu can be sensitized to cisplatin and other antitumor agents by either down-regulating the HER2/neu pathway with an antibody approach (39,40) or restoring wild-type p53 by transfection (41). Results from our present studies are surprising in that they indicate that expression of mutant p53 in the already p53-deficient SK-OV-3 cells induced further resistance to the platinum agent. That mutant p53 was the factor responsible for the increased resistance was inferred from the observation that, at the permissive temperature of 32°C, when p53 functioned as wild-type, the increased resistance (that is, reduced cytotoxicity) was no longer apparent. In contrast, the cytotoxicity of the platinum analog DACH-acetato-Pt was not affected by mutant p53, but significantly increased by wild-type p53.

Our studies using the SK-OV-3 model were facilitated by establishing clones after transfecting tumor cells with a TS-p53 vector that carried a TS mutation at codon 133 (ATG to AGG; M133T). This mutation was initially identified in familial Li-Fraumeni breast sarcoma (42) and was later demonstrated to be temperature-sensitive (G. Lozano, unpublished observation). The two TS clones (TS4 and TS9) were selected for their differential level of expression of mutant p53 (at 37°C), which did not alter the basal expression of HER2/neu. Utilization of these clones in our investigations has demonstrated that there may be a threshold effect for mutant p53 to induce further

resistance to cisplatin; only the clone expressing the higher level of mutant p53 was resistant to the parental drug. This increase in resistance can be reconciled by the understanding that mutant p53 can acquire gain-of-function (43), which promotes cell growth and interferes with apoptosis induced by antitumor agents, including cisplatin (44). Similarly, Blandino et al. (45) have reported that specific p53 mutants confer upon tumor cells a selective survival advantage during chemotherapy. It is reasonable to propose that in our studies the M133T-p53 at 37 °C has gain-of-function properties, which promote cell survival by increasing DNA damage tolerance and, thereby, reducing the cell killing effects of cisplatin. When the clones were influenced to express wild-type p53, the gain-of-function property was eliminated and cytotoxicity of cisplatin was restored to that of control cells. Thus, the wild-type p53 was unable to overcome the inherent cisplatin resistance due to HER2/neu overexpression. In this respect, the effect of increased levels of $p185^{HER2/neu}$ is dominant over wild-type p53 when cisplatin is the DNA-damaging agent. This was consistent with our present observation that restoring wild-type p53 function did not increase basal levels of apoptosis, as has been demonstrated to be the case in some tumor cell lines following transfection with wild-type p53 (46). It is interesting to note, however, that we were unable to generate stable clones following transfection of SK-OV-3 cells with a plasmid containing the full-length wild-type p53 cDNA (data not presented).

Although our data demonstrated that cisplatin resistance was loosely associated with the level of expression of mutant p53, it is possible that mutant p53 may have different effects in individual transfectant clone. This would be consistent with the controversy that exists over the role of wild-type p53 in drug sensitivity or resistance.

For instance, our previous studies have demonstrated that cisplatin resistance was substantially greater in ovarian tumor models harboring wild-type p53 than mutant or null p53 (27). Similarly, other reports have demonstrated that restoring (46,47) and inactivating (17, 48)wild-type p53 function can both enhance tumor cell sensitivity. Thus, there exists the likelihood that other factors influence cellular response to antitumor agents. A molecular factor that has the potential to modulate p53 function is overexpression and/or amplification of the HER2/neu oncogene (21,22). Indeed, recent reports suggest that HER2/neu signaling impacts wild-type p53 activity through both the mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3'-OH kinase pathways (26,49). Our corresponding pilot studies with the wild-type p53 MCF-7 breast tumor model indicate that overexpression of HER2/neu reduced transactivation of p21 in response to cisplatin treatment (50). In the present study, the extent of p21 induction by cisplatin was also low and not consistent with the high degree of serine 15 phosphorylation, which usually contributes to activation of p53 function (16). This suggests that in the SK-OV-3 model, overexpression of HER2/neu inhibits p21 induction by down-regulating p53 function. These results are consistent with those reported by Casalini et al. (22), who demonstrated that although p53 expression was increased following HER2/neu transfection, MDM2 as the p53 transactivation target was also substantially down-modulated. Thus, the dominant effect of $p185^{HER2/neu}$ over p53 is likely related to inhibition of p53 function.

An important finding in our present study is that HER2/neu overexpression only affected p53 function when cisplatin was the inducer of wild-type p53. With the analog DACH-acetato-Pt, p53-mediated transactivation of p21 appeared to be efficient. This

occurred even though serine 15 phosphorylation of p53 was relatively delayed and ser-392 phosphorylation was unaffected by the analog. The data suggest that the p53 activated by cisplatin is not only distinctly different from that activated by DACH-acetato-Pt, but also that its function is differentially affected by HER2/neu overexpression. This insight is novel and demonstrates that the functional p53, activated by a post-translational process that appears to be independent of serine 15 and serine 392 phosphorylation sites, is not inhibited by $p185^{HER2/neu}$ or its signaling pathway. Consequently, the presence of wild-type p53 sensitized TS clones to DACH-acetato-Pt by reducing the threshold of DNA damage tolerance. These findings with the platinum analog are in keeping with our previous observation that this agent can circumvent cisplatin resistance only in the presence of wild-type p53 (27).

An alternative factor that may contribute to the increased cytotoxicity of DACH-acetato-Pt but not that of cisplatin at 32°C may relate to their comparative effects on the $p185^{HER2/neu}$ pathway, as demonstrated by the levels of phosphorylated form of $p185^{HER2/neu}$. Normally, increases in the active phosphorylated form of $p185^{HER2/neu}$ induce the PI3-K/Akt pathway that affects an increase in phosphorylated levels of Akt and results in an anti-apoptotic effect (2). On the other hand, activation of the Erk pathway can induce apoptosis in cells exposed to cisplatin (51). Thus, with cisplatin, the combination of an increase in Akt phosphorylation and a decrease in Erk phosphorylation likely prevent an increase in the cytotoxicity by offsetting the apoptotic effects of wild-type p53. With the reduced or minimal effects of DACH-acetato-Pt on phosphorylated $p185^{HER2/neu}$, Akt and ERK, it is likely that functional activation of $p185^{HER2/neu}$ is

subdued, which may enable the effect of wild-type p53 to predominate and result in increased cytotoxicity.

In conclusion, our study has demonstrated that HER2/neu overexpression differentially affects the function of activated wild-type p53 depending on the agent that is responsible for p53 induction. It is likely that the differential effect is a result of either 1) the qualitative difference in post-translational modification of p53 affected by independent signaling pathways that are activated by cisplatin and DACH-acetato-Pt, 2) the quantitative difference in the degree of activation of the $p185^{HER2/neu}$ pathway or 3) both. The findings support our hypothesis that introduction of wild-type p53 in combination with the analog could be an effective treatment against cells overexpressing HER2/neu.

ACKNOWLEDGMENTS

We thank Dr. Guillermina Lozano (The University of Texas, M.D. Anderson Cancer Center, Houston, TX) for providing the plasmids.

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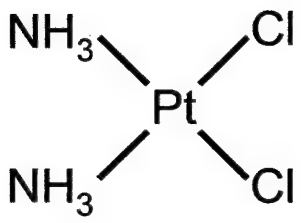
FIGURE LEGENDS

Figure 1 Structures of cisplatin and DACH-acetato-Pt.

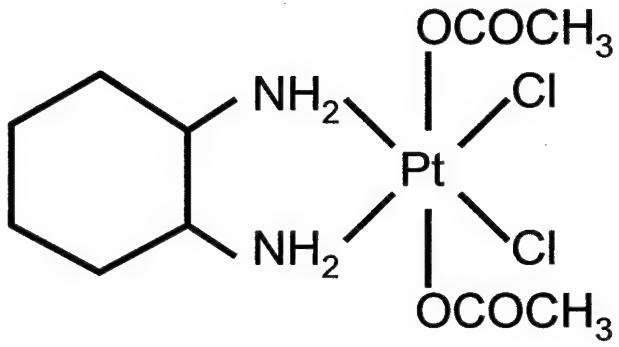
Figure 2 Western immunoblot analysis. A. Basal levels of $p185^{HER2/neu}$ and p53 in SK-OV-3/neo and TS clones at 37°C. MCF-7 cells were used as positive control for p53 and negative control for $p185^{HER2/neu}$. B. Basal levels of p53 and p21 in SK-OV-3/neo and TS clones cultured at 37°C or 32°C for 24 hours. Basal levels of the active phosphorylated $p185^{HER2/neu}$ or the phosphorylated Akt did not change by lowering the incubation temperature to 32°C (data not shown).

Figure 3. Time course of induction of proteins in the TS4 clone treated with 5 x IC_{50} concentration of cisplatin (A) or DACH-acetato-Pt (B) (29.3 μM cisplatin or 29.6 μM DACH-acetato-Pt) at 32°C. The figure indicates results following Western blot analysis.

Figure 4. Quantitation of bands observed following Western immunoblot analysis. The bands observed in Figure 3 were quantified by laser densitometry and plotted over time (• cisplatin; o DACH-acetato-Pt). The serine 392 phosphorylated-p53 is not shown, as it was not detectable in untreated TS4 cells or after treatment with DACH-acetato-Pt.



Cisplatin



1R,2R-DACH-$(Ac)_2Cl_2$-Pt(IV)

Figure 1

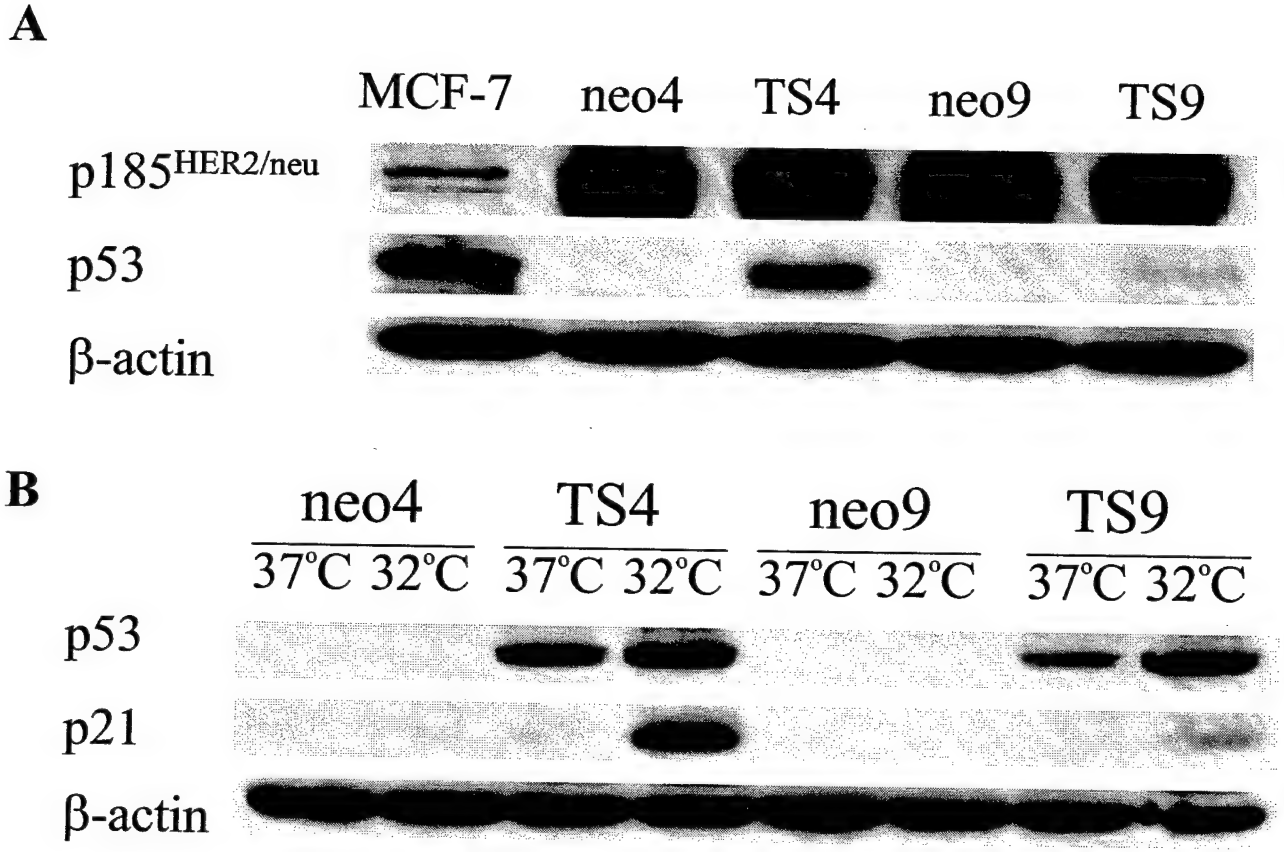
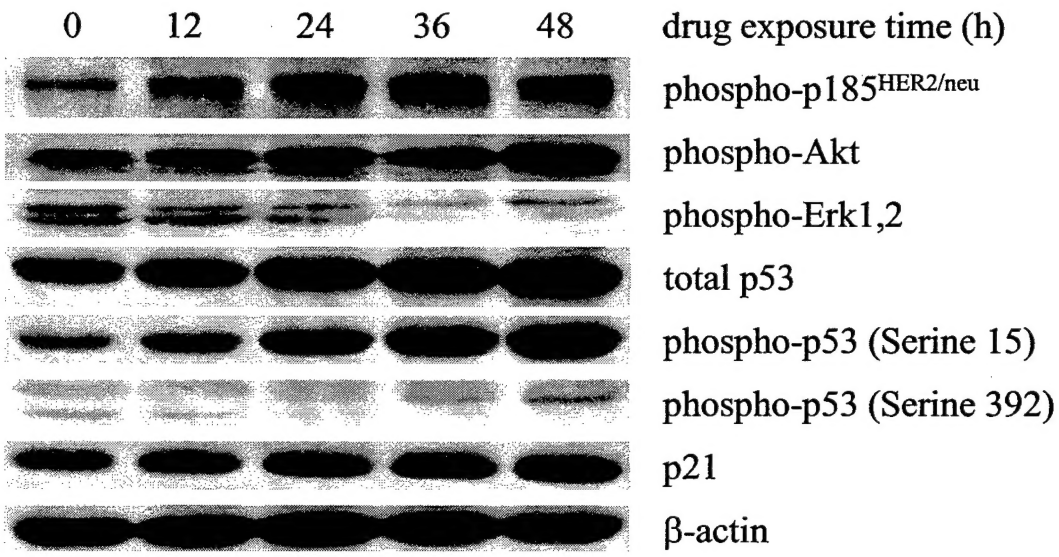

A
MCF-7
neo4
TS4
neo9
TS9
p185HER2/neu
p53
β-actin
B
neo4
TS4
neo9
TS9
37°C 32°C
37°C 32°C
37°C 32°C
37°C 32°C
p53
p21
β-actin

Figure 2

A. cisplatin



B. DACH-acetato-Pt

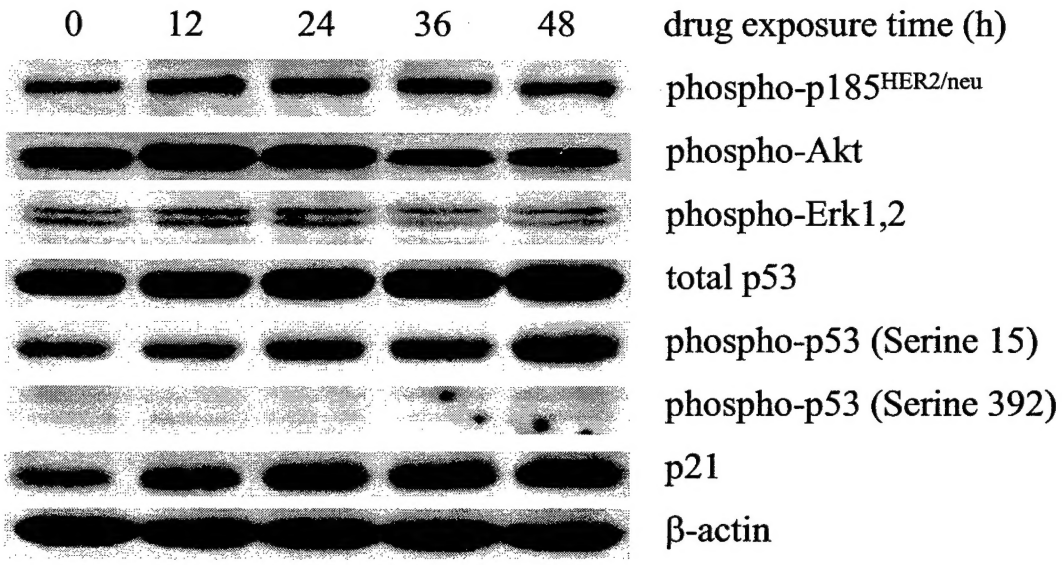


Figure 3

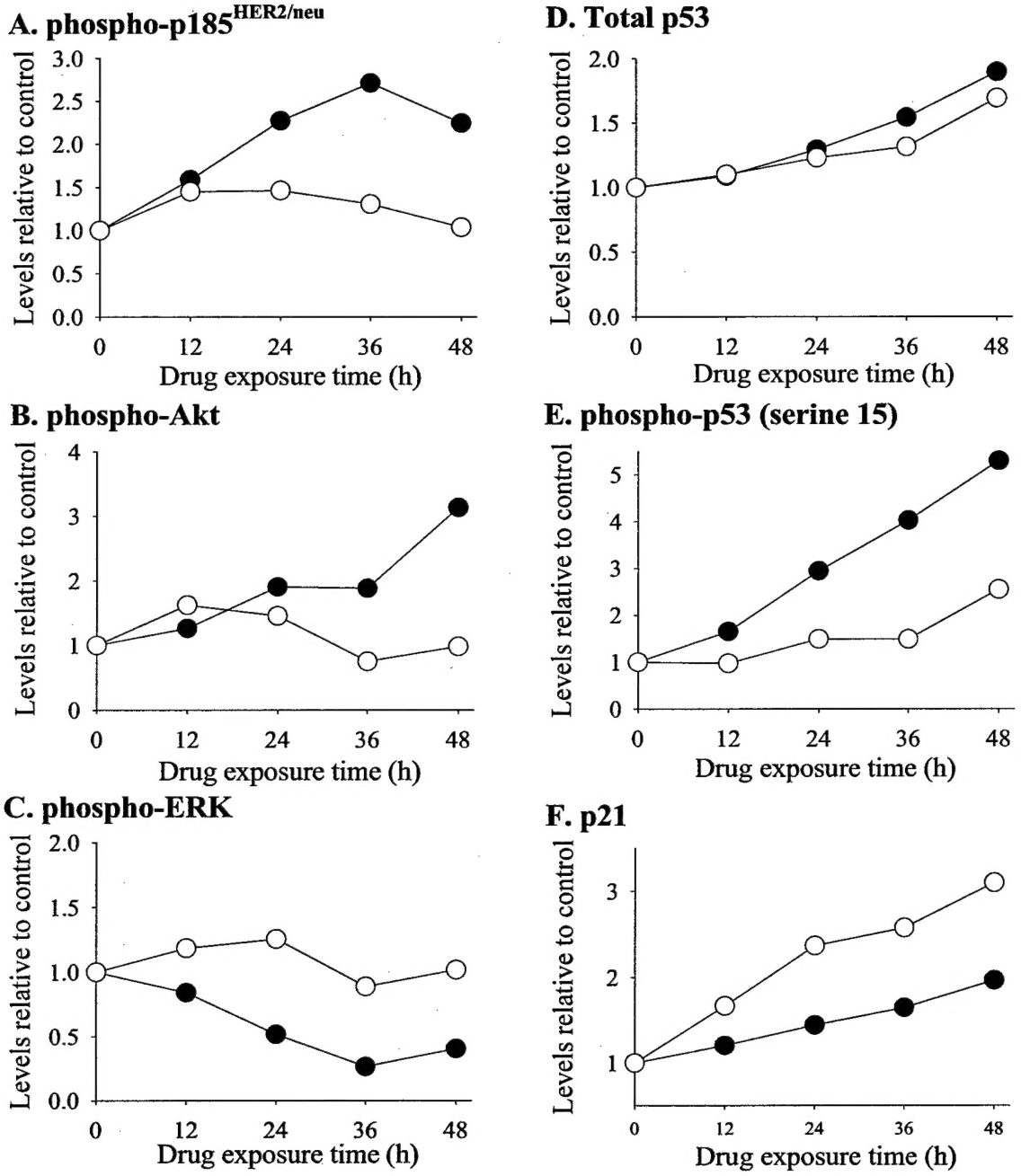

A. phospho-p185HER2/neu
Levels relative to control
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
12
24
36
48
Drug exposure time (h)
B. phospho-Akt
Levels relative to control
4
3
2
1
0
0
12
24
36
48
Drug exposure time (h)
C. phospho-ERK
Levels relative to control
2.0
1.5
1.0
0.5
0.0
0
12
24
36
48
Drug exposure time (h)
D. Total p53
Levels relative to control
2.0
1.5
1.0
0.5
0.0
0
12
24
36
48
Drug exposure time (h)
E. phospho-p53 (serine 15)
Levels relative to control
5
4
3
2
1
0
0
12
24
36
48
Drug exposure time (h)
F. p21
Levels relative to control
3
2
1
0
12
24
36
48
Drug exposure time (h)

Figure 4

Table 1. Effect of TS p53 transfection on the cytotoxicity of SK-OV3 cell line

	37 degrees		32 degrees	
clone	cisplatin	DACH-acetato-Pt	cisplatin	DACH-acetato-Pt
neo4	0.84 ± 0.24*	1.58 ± 0.29	5.94 ± 1.08	14.3 ± 3.1
TS4	1.63 ± 0.12**	2.19 ± 0.39	5.86 ± 0.82	5.91 ± 1.35**
TS4/neo4 ratio	*1.94*	*1.39*	*0.99*	*0.41*
neo9	2.33 ± 0.61	11.1 ± 1.5	4.27 ± 0.82	22.7 ± 5.6
TS9	2.16 ± 0.40	8.78 ± 2.43	3.55 ± 1.76	4.63 ± 0.24**
TS9/neo9 ratio	*0.93*	*0.79*	*0.83*	*0.20*

*Mean ± SD; **$P<0.05$, vs. neo with *t*-test; n=3

Table 2. Effect of TS p53 transfection on the cytotoxicity of SK-OV3 cell Boichemical pharmacology of cisplatin and DACH-acetato-Pt in SK-OV3/clone4

	37 degrees		32 degrees	
clone	cisplatin	DACH-acetato-Pt	cisplatin	DACH-acetato-Pt
Platinum uptake (ng Pt/mg protein)				
neo4	81.8 ± 33.8*	31.8 ± 12.1	46.4 ± 21.4	19.2 ± 5.4
TS4	81.5 ± 32.3	31.8 ± 13.7	45.1 ± 21.1	19.0 ± 6.8
DNA adducts (ng Pt/mg DNA)				
neo4	39.8 ± 12.8	7.7 ± 1.4	20.8 ± 10.7	7.9 ± 2.5
TS4	30.1 ± 10.6	7.1 ± 2.1	19.7 ± 13.4	8.6 ± 3.2
DNA damage tolerance (ng Pt/mg DNA at IC_{50})				
neo4	0.33 ± 0.09	0.12 ± 0.02	1.24 ± 0.22	1.13 ± 0.24
TS4	0.49 ± 0.03**	0.16 ± 0.03	1.15 ± 0.16	0.51 ± 0.12**

*Mean ± SD; **$P<0.05$, vs. neo4 with t-test, n=3-4